Route of Entry-Dependent Blocks to Retroviral Replication

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I, Eleanor Ruth Gray, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Restriction factors are endogenous cellular proteins that block retroviral replication at specific points in the life cycle. Those identified so far include Fv1, Trim5α and TrimCyp. Their characterisation has extended knowledge of retroviral and cellular functions, and has added a new branch to innate immunity.

Retroviral susceptibility to Fv1 and Trim5α is determined by its capsid, and is manifested in a pre- (Trim5α, TrimCyp) or post- (Fv1) reverse transcription block to replication. Other blocks to replication have been postulated. For example, a novel anti-viral factor, Lv2, is thought to block replication of several primary isolates of HIV-2 in some cell lines.

Knowledge of the early steps of virus replication, between entry and nuclear import, is critical to understanding restriction. The intention of the studies described in this thesis was to determine whether alternative routes of virus trafficking might affect susceptibility to Fv1 and Trim5α, as well as to the putative Lv2. A system of two receptors was used, Tva800 and Tva950; both permit entry via ASLV envelope protein, but take the virus into the cell by two different endocytic mechanisms.

The pathways traversed after binding to Tva800 and Tva950 were investigated and shown not to reroute virions around restriction mediated by Fv1 and Trim5α. When virus titration curves were analysed, a distinctive pattern emerged suggesting that entry via Tva800, but not Tva950, requires engagement of more than one receptor-envelope pair.

The block to replication caused by the putative factor Lv2 was also analysed. It was concluded that a combination of low surface CD4 expression and poor receptor engagement are the cause of low viral titres in some cell lines, rather than a cellular anti-viral factor per se.

Acknowledgements

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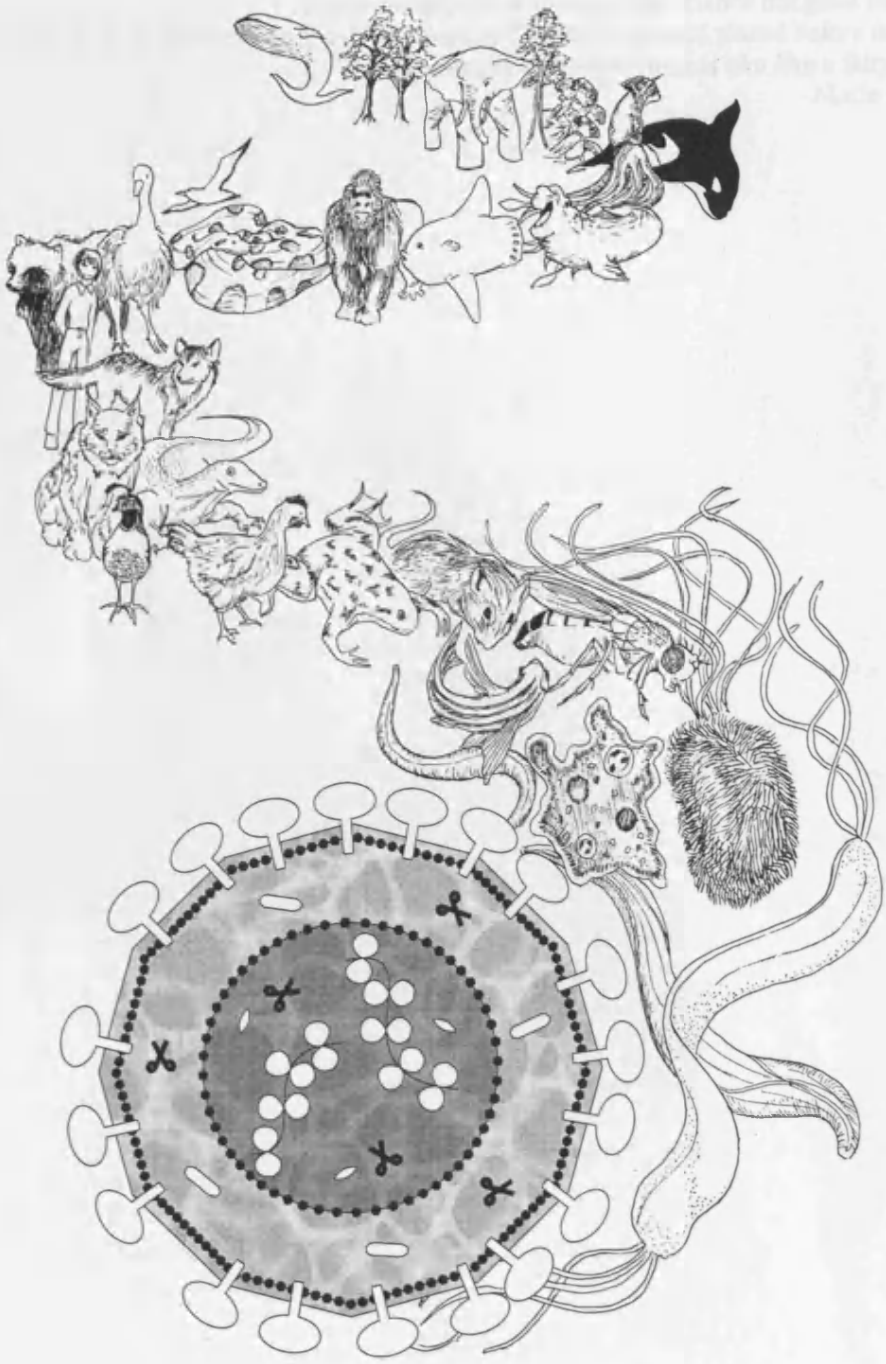
The support of my family has been so important to me. Thanks to Morag and Serge for being so encouraging and for the beautiful picture – this is for you (Simpson *et al.* 2006); I told you I'd manage it somewhere! Thank you to Julia and Hazel, and to Cameron and Margaret. And thanks are finally and most importantly due to my parents, for rock-solid faith in me, and for being themselves so inspirational.



I am among those who think that science has great beauty.
A scientist in his laboratory is not only a technician: he is also a child placed before natural phenomena which impress him like a fairy tale.
Marie Curie

May the words of my mouth and the meditation of my heart be pleasing in your sight...
Ps 19v14

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Abbreviations

AIDS	acquired immunodeficiency syndrome
APOBEC	apolipoprotein B mRNA-editing enzyme catalytic polypeptide
ASLV	avian sarcoma and leucosis virus
CA	capsid
CD	cluster of differentiation
CMV	cytomegalovirus
CXCR	CXC motif chemokine receptor
d800	*Mus dunni* cells, expressing Tva800
d950	*Mus dunni* cells, expressingTva950
DMEM	Dulbecco's modified Eagle's medium
DN	dominant negative
DNA	deoxyribonucleic acid
DTT	dithiothreitol
ds	double stranded
EEA1	early endosome antigen 1
ECL	enhanced chemiluminescent
eGFP	enhanced green fluorescent protein
Env	envelope
ER	endoplasmic reticulum
ERV	endogenous retrovirus
Fv1	Friend virus restriction 1
Gag	group specific antigen
GPI	glycosylphosphatidylinositol
GPI-AP	glycosylphosphatidylinositol-anchored protein
HA	haemagglutinin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	human immunodeficiency virus
Hr	hour
IN	integrase
IRES	internal ribosome entry site
LacZ	β-galactosidase
LDL	low density lipoprotein
LTR	long terminal repeat
Lv1/Lv2	lentivirus restriction 1/2
MA	matrix
mac	rhesus macaque
MCR/MCN	molecular clone restricted/non-restricted
MHR	major homology region
Min	minute
ml	mililitre
µl	microlitre
MLV	murine leukaemia virus
MOI	multiplicity of infection
MSD	membrane-spanning domain
NC	nucleocapsid
Nef	negative factor
NMR	nuclear magnetic resonance

ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
pbs	primer binding site
PIC	pre-integration complex
Pol	polymerase
PR	protease
(q)PCR	(quantitative) polymerase chain reaction
Rab	Ras-related in brain
Refl	restriction factor 1
RNA	ribonucleic acid
RSV	Rous sarcoma virus
RT	reverse transcriptase
SDS	sodium dodecyl sulphate
Secs	seconds
SiRNA	small interfering ribonucleic acid
SIV	simian immunodeficiency virus
smm	sooty mangabey monkey
ss	single stranded
SU	surface subunit (of envelope protein)
SV40 ori	origin of replication, SV40 promoter
TEMED	N,N,N',N'-Tetramethylethylenediamine
TBE	Tris/borate/EDTA buffer
TM	transmembrane region (of envelope protein)
Trim	tripartite motif
Tv(a)	tumour virus (a)
Vif	viral infectivity factor
Vpr	viral protein R
Vpu	viral protein u
Vpx	viral protein x
VSV	vesicular stomatitis virus
YFP	yellow fluorescent protein

Chapter 1

Introduction

Members of the family *Retroviridae*, which is in the class of Viruses, undergo a stage in their infectious cycle whereby their single stranded RNA genome is converted into double stranded DNA. This provides an exception to the central dogma of molecular biology as initially proposed by Francis Crick in 1958, which is that the normal direction of flow of information in biology is from DNA→RNA→protein (Crick 1958; Crick 1970). Retroviruses have long been efficient invaders of many species of plants and animals, and have left glimpses of their co-evolution alongside hosts in the now defunct viruses scattered as junk DNA across genomes (Griffiths 2001; Villesen et al. 2004).

Viruses were first discovered in 1892 when the Russian botanist Iwanowski found that the causative agent of tobacco mosaic disease was small enough to pass through a ceramic filter that would trap bacteria (Iwanowski 1892), although the idea of a disease causing agent that was separate from bacteria was defined more precisely a few years later by Beijerinck (Beijerinck 1898). Retroviruses were first found not long after during investigations of diseases in chickens. Ellerman and Bang demonstrated that leucosis in chickens was caused by a virus (avian leucosis virus, ALV) (Ellerman and Bang 1908), and Rous showed that sarcoma in chickens could be transmitted by a cell-free agent, named Rous sarcoma virus (RSV) (Rous 1911). While these viruses were recognised to have an RNA genome, it was thought that like a picornavirus (Landsteiner and Levaditi 1909; Baltimore and Franklin 1963; Warner et al. 1963), the genome was the direct template for transcription of mRNA, or itself was used directly for translation of viral proteins. However, experimental studies failed to provide evidence of necessary intermediates such as double-stranded RNA, and additionally threw up confounding facts such as the sensitivity of retroviral replication to inhibitors of DNA synthesis (Temin 1963; Temin 1964). Finally, these data were drawn together when in 1964 Temin put forward the theory that these viruses could reverse the standard flow of information, and synthesise DNA from

RNA (Temin 1964). This idea was met with derision, and only rendered acceptable when reverse transcriptase was discovered in retroviral virions in 1970 (Baltimore 1970; Temin and Mizutani 1970).

The discovery that retroviruses can acquire cellular genes which after subsequent integration events and stepwise changes in cellular regulation can be oncogenic, raised the stakes for discovery of a cancer-causing human retrovirus, and human T-cell leukaemia virus-1 (HTLV-1) was isolated in 1980 (Poiesz et al. 1980). Once definitively linked with a human disease, interest in retroviruses was ensured. Much of the continuing research since has focussed on the currently most well known retrovirus, human immunodeficiency virus (HIV). It was discovered as the causative agent of a mysterious immune deficiency first seen in the early 1980s, and rapidly identified as a retrovirus (Barre-Sinoussi et al. 1983; Gallo et al. 1984). In 2007 acquired immunodeficiency syndrome (AIDS) caused by HIV-1 or HIV-2 was a factor in the deaths of 2.1 million people, 1.6 million in sub-Saharan Africa alone, and up to 36 million further potentially infected (Joint United Nations Programme on HIV/AIDS. 2007).

1.1 Virus Taxonomy

Retroviruses were initially classified according to the morphology of the virion core as seen under the electron microscope, so that those with similar core structures were grouped together. Genera are now clustered according to genomic organisation and the timing of reverse transcription, as well as core morphology into two subfamilies, and are as follows (with examples in parentheses): the orthoretrovirinae comprising alpharetroviruses (avian sarcoma and leucosis virus, ASLV); betaretroviruses (mouse mammary tumour virus); gammaretroviruses (murine leukaemia virus, MLV); deltaretroviruses (HTLV); epsilonretroviruses (walleye dermal sarcoma virus); lentiviruses (HIV-1); and the subfamily spumaretrovirinae, which contains only spumaviruses (human foamy virus) (Hunter *et al.* 2000; Linial *et al.* 2005). Within these species further divisions can be made. For example, HIV-1 worldwide can be organised into genetically distinct subtypes, where virions in a subtype differ from the other subtypes in amino acid composition by at least 20% in the envelope region, and 15% in the Gag region (Robertson et al. 2000; Levy 2007).

1.2 Genome Organisation

Outlines of two specific retroviral genomes are shown in figure 1.2, and the corresponding proviral DNA, mRNAs and proteins that would be produced from the Moloney MLV genome.

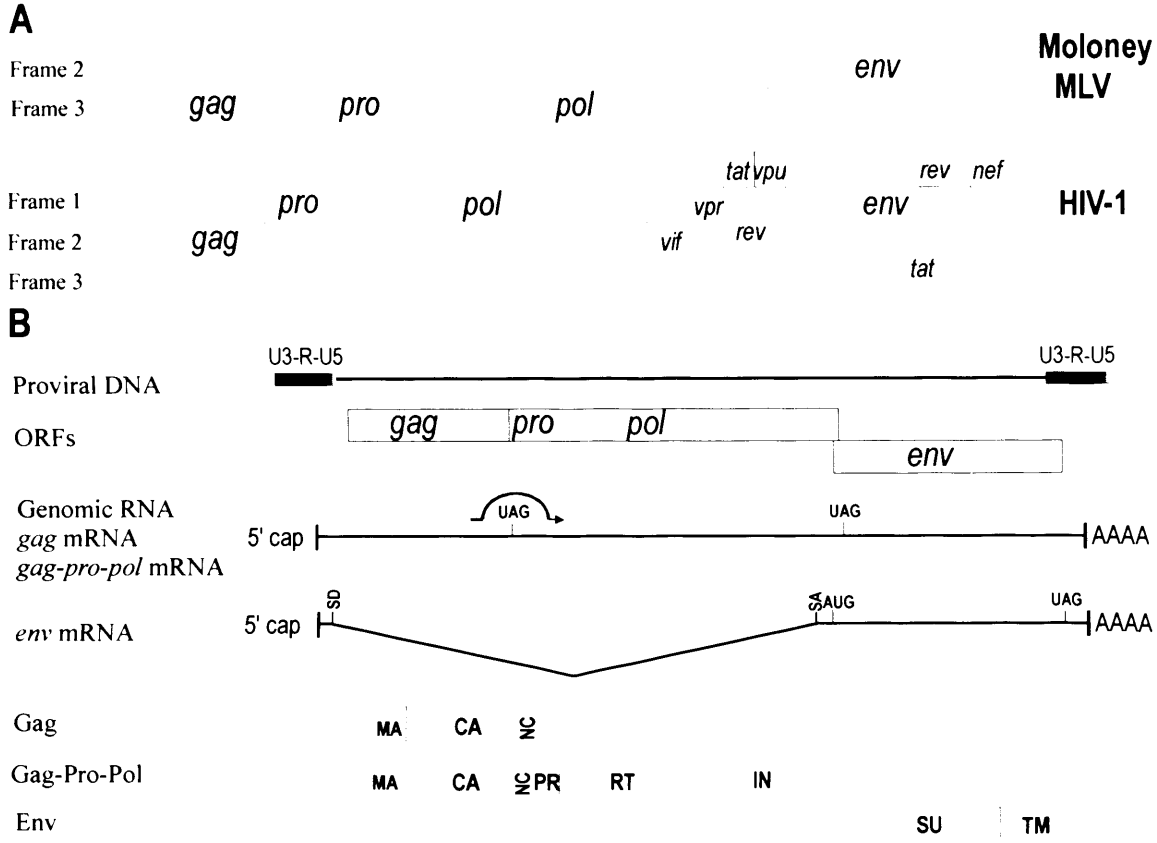


Fig. 1.2. MLV and HIV-1 genomes. MLV and HIV-1 open reading frames are shown in A, MLV genes, mRNAs and proteins are shown in B. The proviral DNA has the U3-R-U5 sequence, the long terminal repeat, at both ends. Gag, Gag-pro-pol and Env mRNA and genomic RNA for progeny virions are transcribed from the integrated DNA. The stop codon UAG shown is read through (suppressed) to generate *gag-pro-pol* mRNA. Key sequences are found at the 5' and 3' ends, which facilitate the remarkable transcription strategy of the virus. Initial translation is of the polyproteins shown at the bottom, which are later cleaved into their constituent parts. Proteins are indicated by the standard two letter nomenclature (Leis et al. 1988). SD/SA, splice donor, splice acceptor. Adapted from (Muesing et al. 1985; Fields et al. 2007)

The genome structure differs according to whether the retrovirus is considered simple (alpha-, beta- and gammaretroviruses) or complex (delta-, epsilon-, lenti- and spumaviruses). The genomes of the former encode the three genes, *gag*, *pol* and *env*, whereas the latter additionally encode many open reading frames (ORFs) of accessory

genes. Despite the epithet 'accessory', most of these are essential for infectivity, at least in certain cell types and *in vivo* infection (Balliet et al. 1994; Anderson and Hope 2004). For all retroviruses, different mRNAs encoding single or combinations of these proteins are produced by splicing reactions. For MLV, a simple retrovirus, there is just one splicing reaction that removes the entirety of *gag*, most of *pol*, and produces a transcript of just *env* (Shinnick et al. 1981). For HIV there are many more splicing possibilities between different ORFs created by use of several alternative splice sites (Arrigo *et al.* 1990; Guatelli *et al.* 1990; Schwartz *et al.* 1990a). These allow control of production of different proportions of the viral proteins, and through the accessory protein Rev, also control of timing of production (Malim et al. 1989; Katz and Skalka 1990). As the genome is produced by the host cell it has several standard modifications that would be present on host mRNAs, for example, it will be capped at the 5' end, and polyadenylated at the 3' end (Green and Cartas 1972; Furuichi et al. 1975; Reddy et al. 1980). In the mature retrovirus two copies of the genome will be packaged. During transcription, both copies can serve as a template for reverse transcription, leading to deletions, insertions, and duplications. Template switching between the two copies can also lead to exchange of genetic markers between clades, when one cell becomes infected with two viruses, resulting in inter-subtype recombinants. This causes problems when drug resistance mutations in genes are exchanged.

Once processed, translation of pro and pol is controlled by sequences at the *gag-pro* and *pro-pol* junctions. A stop codon at the end of *gag* may be read through to produce Gag-Pro-Pol (e.g. MLV, (Yoshinaka et al. 1985), or a frameshift can occur, which is when the ribosome slips back a nucleotide due to both stalling during reading of a UUUUUUA length and the encounter with secondary structures in the mRNA (Hung *et al.* 1998). This also produces a Gag-Pro-Pol polyprotein (e.g. alpharetroviruses, (Jacks and Varmus 1985). In some retroviruses (e.g. mouse mammary tumour virus, and HTLV-1) two framshifts are required as *gag*, *pro* and *pol* are all in separate reading frames. The efficiency with which the slippage or frameshifting occurs determines the ratios of shorter to full length polyproteins. These initial products are large precursor proteins, cleaved into their constituent parts by the viral protease at specific points later in the life cycle (Yoshinaka and Luftig 1977).

1.3 Structure

While retroviruses have been separated into their genera partly on the basis of differences in structure, there are many common features between them. A typical structure is shown in figure 1.3.

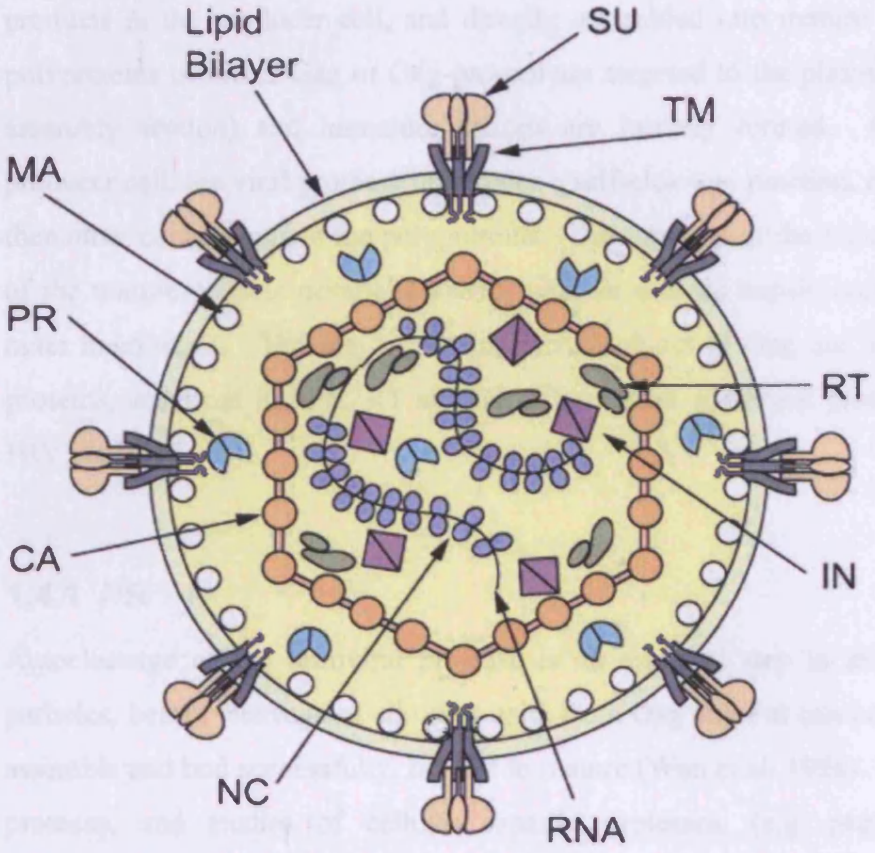


Fig. 1.3 A schematic structure of a typical retrovirus particle. The main components, encoded by the retroviral genome are shown. Other host proteins may also be present. Adapted from (Coffin et al. 1997).

The structure can broadly be considered in two parts. The inner part is the core, contained by the polymerised capsid protein (CA). The two strands of genomic RNA are covalently linked and found in the core, associated with nucleocapsid (NC), in a tight complex (Badorrek *et al.* 2006). Other essential retroviral proteins, such as integrase (IN) and reverse transcriptase (RT) are also found in the core. Protease (PR) is found both inside and outside the core. Around the core is a shell, approximately spherical, made of matrix protein (MA), which is itself bounded by a phospholipid bilayer. The Env protein is made up of SU (surface protein) and TM (transmembrane

protein), and is inserted into the outer layer; this is acquired by the virion as it buds through the cell membrane, exiting the cell.

1.4 Proteins

The structural proteins that make up the capsid core are not synthesised as finished products in the producer cell, and directly assembled into mature viruses. Rather, polyproteins of either Gag or Gag-pro-pol are targeted to the plasma membrane (see assembly section) and immature virions are initially formed. Once out of the producer cell, the viral protease undergoes a self-cleavage reaction, excising itself and then other components of the polyproteins. The structure of the virion changes to that of the mature particle described above, with an ordered capsid core and a spherical outer membrane. The major proteins processed out of Gag are MA, CA, and NC proteins, and from Pol PR, RT and IN. There is an additional protein cleaved from HIV Gag, p6.

1.4.1 PR

Autocleavage of the retroviral protease is an essential step in maturation of viral particles, before cleavage of all other units from Gag and Pol can occur. PR mutants assemble and bud successfully, but fail to mature (Wan et al. 1996). PR is an aspartic protease, and studies of cellular aspartic proteases, (e.g. pepsin) have aided characterisation. Many of these proteins have regulatory switches, however, this switch is usually between completely on and completely off mechanisms, whereas PR must have some low level of activity in order to execute the initial autocleavage. PR itself matures during auto-processing. The first stage of PR maturation is dimerisation (Wondrak and Louis 1996; Wondrak et al. 1996), which would presumably be more likely when the polyprotein is at high concentration, as is the case in the immature particle. Regions upstream of PR also regulate autoprocessing (Gatlin *et al.* 1998a; Gatlin *et al.* 1998b). Inhibitors of mature PR fail to block cleavage of the Gag-pol between NC and p6, indicating that it can be cleaved by immature PR. Mature PR, however, cannot cleave this bond *in vitro*, indicating that it is probably the first bond to be cleaved as failure to do so would result in an incompletely processed polyprotein (Lindhofer et al. 1995). Sequences in the

transframe region just upstream of PR, comprising a transframe peptide (TFP) and p6* are important for PR regulation. Deletion studies of p6* showed that it has an inhibitory effect on PR activity (Partin et al. 1991; Tessmer and Krausslich 1998). A tripeptide just upstream of p6* in the TFP octapeptide is a potent inhibitor of PR (Louis et al. 1998), and may be a key element in the timing of regulation of activation and subsequent deactivation of PR, which is necessary once the viral particle has matured.

1.4.2 RT

RT confers on the virus the ability to transcribe dsDNA from their ssRNA genomes. The structure of the enzyme varies between retroviruses. MLV RT is a monomer with both DNA polymerase function and RNase H activity (Tanese and Goff 1988). ASLV RT is a heterodimer due to incomplete cleavage at the RT-IN boundary leading to one subunit of DNA polymerase-RNAse H-IN, and one of DNA polymerase-RNase H. The DNA polymerase uses either RNA or DNA as template, and a primer of host tRNA (Gilboa *et al.* 1979). As a polymerase it is slow, dissociates frequently, and pausing is caused by secondary structures present in the templates (Harrison et al. 1998). RNase H acts on RNA in an RNA-DNA duplex, degrading the RNA so that the remaining strand can act as a template for the DNA polymerase.

1.4.3 IN

Entry of proviral DNA into the nucleus and its subsequent integration into the host chromosome is an essential step for retroviral replication. Integration is primarily mediated by integrase (Panganiban and Temin 1984; Quinn and Grandgenett 1988), with other host proteins also playing a role (Bowerman et al. 1989; Turlure et al. 2004). Integrase enzyme is usually found as a dimer, each monomer consisting of three domains; one for Zn^{2+} binding, a catalytic core domain, and a C-terminal domain. It catalyses the removal of two nucleotides from the 3' ends of a proviral DNA, and the attack and integration of this prepared DNA on a phosphodiester bond in the host chromosomal DNA (Fujiwara and Mizuuchi 1988; Brown et al. 1989; Craigie et al. 1990). While integration can theoretically occur anywhere in the genome, sites that are transcribed seem to be targeted for HIV, MLV and ASLV

(Maxfield *et al.* 2005). However, the extent of this preference differs. MLV also shows a preference for integration near transcriptional start sites, whereas ASLV does not, and only has a weak preference for active genes. The non-random nature of integration strongly suggests the involvement of cellular factors, and indeed the protein p75/LEDGF (lens-derived epithelium growth factor) has been highlighted as essential (section 1.5.5) (Cherepanov et al. 2003; Llano et al. 2006). Genes that are very highly transcribed seem to be selected less than those that are transcribed at a low level (Maxfield et al. 2005).

1.4.4 MA

The matrix protein is made of a monomer of helices surrounding a hydrophobic core. Sequences in MA are key to the targeting of Gag to the membrane during assembly of nascent viral particles. If the M domain, which is found at the N-terminus of MA, is mutated, assembly is defective, but it can be rescued with coexpression of wild type MA. Targeting requires myristoylation of the M domain (addition of a 14-carbon fatty acid), and blockage of this step abolishes viral assembly (Gottlinger et al. 1989; Yuan et al. 1993). Depending on the conformation state of MA, this myristoyl group can be either exposed or sequestered. Binding of $PI_{4,5}P_2$ to MA promotes an exposed conformation, revealing a cluster of basic residues in MA that binds to the membrane (Zhou *et al.* 1994). HIV MA also has a nuclear localisation signal that may be important for nuclear import, but it is not found in the pre-integration complex of other retroviruses.

1.4.5 NC

Nucleocapsid is a small, basic protein found associated with the RNA genome in the retroviral core that binds a Zn^{2+} ion. Alteration or deletion of this sequence causes aberrations in RNA packaging (Aldovini and Young 1990). NC is also important in early stages of reverse transcription as it promotes the annealing of primer tRNA to the binding site, and strand transfer (Rong *et al.* 1998c).

1.4.6 CA

The capsid protein is the largest of the Gag proteins, and forms a polymerised shell enclosing the core, a feature unknown in many other RNA viruses. In all retroviruses (except spumaviruses) the one highly conserved sequence in Gag, the major homology region (MHR), is found in CA and is important for virion assembly (Mammano et al. 1994; Craven et al. 1995). The structure of CA has been solved for several viruses (e.g. HIV (Gamble et al. 1996; Gamble et al. 1997; Berthet-Colominas et al. 1999). Structurally, CA is made up of two domains, an N-terminal two-thirds and C-terminal third, connected by a linker. The N-terminal domain of HIV-1 is mostly alpha-helical with an exposed loop that cyclophilin A binds, and an N-terminal β-hairpin. The MHR is found in the C-terminal domain.

In polymerised form the capsid core can be spherical (MLV), coffin- (HIV) or bar- (Mason-Pfizer monkey virus) shaped. The role of capsid *in vivo* requires a balancing of two seemingly opposite requirements; the core must be stable enough to keep the viral genetic material safe during viral egress, maturation, binding and entry, and yet also able to dissociate as soon as required in the infectious cycle after entry into a naïve cell. The capsid shell only forms after maturation, and after this point becomes relatively unstable in detergents, indicating that it is then able to break down quickly after entry (Wiegers et al. 1998). In the immature virion the core is round, with a relatively sparse centre. During maturation Gag is cleaved into its constituent parts and the core condenses, with capsid arranged as hexamers in a lattice (Mortuza et al. 2004; Ganser-Pornillos et al. 2007). Despite the apparent importance of CA in maturation, mutations that remove almost this entire domain in ASLV do not cause loss of budding.

A renewed focus on CA recently has come as a result of its role as a determinant for restriction by Fv1 and Trim5α. The interaction of these factors with CA is discussed in later sections.

1.4.7 p6

The lentiviral p6 is a 6kDa protein is found at the C-terminus of Gag, and is a pro-rich protein that functions late in the infectious cycle, during the assembly process of HIV-

1, incorporating vpr and vpx into the virions (Lu et al. 1993). Particles with defective p6 remain tethered at the cell surface (Gottlinger et al. 1991). PT/SAP domains in p6 also promote viral release by binding to Tsg101, a component of the cellular machinery that mediates sorting into, and biogenesis of, multi-vesicular bodies (Morita and Sundquist 2004). Another sorting protein, ALIX, can also bind to p6 of HIV, but this interaction is not critical for release (Strack *et al.* 2003).

1.4.8 Env

The *env* gene is found at the 3' end of the genome, and is produced from a singly spliced mRNA, from which either the entire *gag-pro-pol* coding regions have been removed (several retroviruses including MLV (Shinnick et al. 1981)), or the first 6 codons of *gag* spliced to *env*, as is the case for ASLV (Hunter *et al.* 1983). Levels of Env vary amongst the retroviruses, from equimolar (Gag:Env) in gammaretroviruses to much lower levels in the mature lentiviruses (Grief et al. 1989; Chertova et al. 2002; Zhu et al. 2003; Yuste et al. 2005). During production the nascent protein is inserted into the ER after which it will be glycosylated and further modified in the Golgi before being trafficked to the plasma membrane, as would occur for membrane proteins of the host cell. Env must oligomerise and fold correctly (e.g. ASLV Env forms trimers (Einfeld and Hunter 1988)). It is important that Env does not interact with the receptor when it is in the process of being correctly constructed and transported out to the cell surface. Different viruses have evolved different mechanisms to ensure that this does not occur. For example, ASLV produces levels of Env that are vastly in excess of the receptor (Bates et al. 1993; Young et al. 1993), and HIV-1 Vpu binds to the cytoplasmic domain of CD4 and induces its down-regulation (Willey et al. 1992).

Cleavage of the envelope protein into SU and TM occurs later, but while Env is still in the Golgi, and is mediated by a host cell protease (Decroly et al. 1994; Gu et al. 1995). For ASLV, cleavage occurs at a di-basic pair, and leaves TM with a hydrophobic N-terminus, the location of the fusion peptide (Dong et al. 1992). Env is then capable of mediating fusion (Freed et al. 1995). Once at the cell surface, Env proteins remain in the multimers of the SU-TM dimer. Under the electron microscope they appear as knobbly protrusions from the surface of the virion. During binding and

entry several rearrangements of intra- and inter-SU and TM bonds takes place, and HIV-1 entry may involve SU removal.

1.4.9 Accessory proteins

In addition to those described above, a number of several further retrovirally encoded proteins are also found in HIV particles to varying degrees (Robert-Guroff *et al.* 1990; Schwartz *et al.* 1990b). Mutations in these genes do not affect viral replication in some cultured cells, but these proteins are essential *in vivo* for productive replicative cycles.

Vif (virus infectivity factor) interacts with APOBEC3G during assembly. This aspect is discussed later, along with other restriction factors.

Vpr (viral protein r) is associated with the pre-integration complex, and may play a role in its nuclear localisation (Heinzinger et al. 1994). It causes cell cycle arrest and apoptosis, and also has a role in facilitating efficient infection of non-dividing cells (Andersen and Planelles 2005). An association with the cellular protein DDB1 is necessary for the G_2 arrest, and this association prevents DDB1 from carrying out its normal cellular role of repairing DNA damaged by UV. DDB1 also targets some regulatory proteins of the cell cycle for degradation, forming a ubiquitin ligase complex with Cul4A and Roc1 (Schrofelbauer et al. 2007). Vpr is found in high copy number in virions, persisting in association with proviral DNA up to and into the nucleus, which has made fusions of Vpr and fluorescent proteins useful in visualising the viral core after entry (McDonald et al. 2002).

Vpu is found in HIV-1 and chimpanzee SIV, and bears homology to the potassium ion channel, TASK-1 (Hsu et al. 2004). Vpu is not found in viral particles, and negative mutants produce viral proteins normally, but are defective in virion production in some cell types (Klimkait et al. 1990). Vpu induces CD4 degradation by binding to the cytoplasmic tail of CD4 in the ER, inducing its degradation (Willey et al. 1992). This prevents re-infection of the infected cell. It also counteracts the anti-viral activity of a set of cellular proteins (or tetherins), including CD317, which attach mature viral particles to the cell surface (Neil et al. 2008).

Vpx is an additional gene found in viruses of HIV-2/SIV_{smm}/SIV_{mac} lineage. It plays a similar role to vpr.

Nef (negative factor) is core-associated, and packaged into virions. It is essential for sustaining viral production *in vivo* and acts by down regulating antigenic and viral receptor cell-surface molecules, enhancing viral infectivity and modulating cellular pathways (Schaeffer et al. 2001; Qi and Aiken 2007).

1.5 Virus Infectivity Cycle

Retroviruses must pass through a formidable array of defences before they can successfully enter the cell and complete a replicative cycle. First of all they must traverse the physical barriers; the viral core has to access the cytoplasm, and then, while disassembling, pass through the thick barrage of proteins and webs of membranes that make up the cytoplasm towards the nucleus, and the newly reverse transcribed genetic material must finally traverse the nuclear membrane and outmanoeuvre the histones and other DNA-associated proteins to integrate into the host DNA. Secondly, they must overcome the cell's biological defences. These can be intrinsic defences or part of the adaptive immune system. Different viruses have evolved many strategies to avoid both physical and biological defences that the cell raises.

The cycle can be broadly split into two stages. In the first, the virus enters the cell, reverse transcribes and integrates its genetic material into the host DNA. These processes are extraordinary for the cell, and use co-opted cellular proteins directed by viral machinery. In the second half of the cycle, progeny virions are produced, assemble and exit the cell, using the routine cellular mechanisms of translation, transcription and transport. A basic outline of the main steps is shown in figure 1.5.

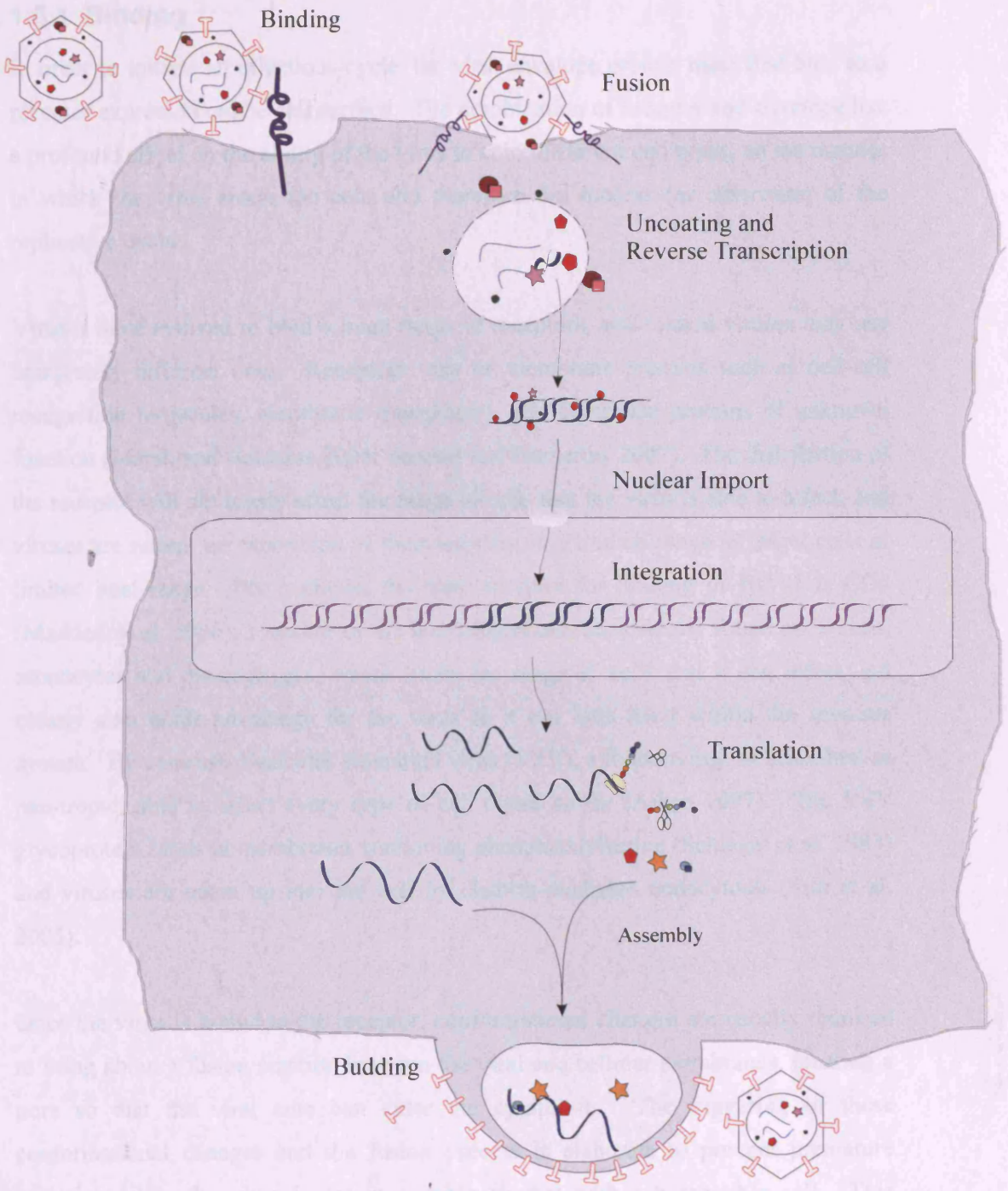


Fig. 1.5 A schematic of the retroviral infectious cycle. In order to replicate, viruses must enter cells, reverse transcribe their RNA genome, then integrate the newly synthesised DNA into the host chromosomal DNA. From there cellular mechanisms transcribe, translate and partly process the retroviral proteins. Virions assemble and bud from the plasma membrane, completing the cycle.

1.5.1 Binding

In order to initiate an infectious cycle, the viral envelope protein must first bind to a receptor expressed on the cell surface. The combination of receptor and envelope has a profound effect on the ability of the virus to enter different cell types, on the manner in which the virus enters the cell, and therefore the success (or otherwise) of the replicative cycle.

Viruses have evolved to bind a huge range of receptors, and related viruses may use completely different ones. Receptors may be membrane proteins such as cell-cell recognition molecules, membrane transporters, and some use proteins of unknown function (Marsh and Helenius 2006; Stewart and Nemerow 2007). The distribution of the receptor will obviously affect the range of cells that the virus is able to infect, and viruses are reliant on expression of their receptor on a limited range of target cells or limited host range. For example, the main receptor for binding of HIV-1 is CD4 (Maddon et al. 1986), a marker of the immunoglobulin superfamily found on T-cells, monocytes and macrophages, which limits the range of cells that it can infect, but clearly also holds advantage for the virus as it can hide itself within the immune system. By contrast, Vesicular stomatitis virus (VSV), a rhabdovirus, is described as pan-tropic, able to infect every type of cell tested so far (Aiken 1997). The VSV glycoprotein binds to membranes containing phosphatidylserine (Schlegel et al. 1983) and viruses are taken up into the cell by clathrin-mediated endocytosis (Sun et al. 2005).

Once the virus is bound to the receptor, conformational changes are usually required to bring about a fusion reaction between the viral and cellular membranes, creating a pore so that the viral core can enter the cytoplasm. The triggering of these conformational changes and the fusion process is elaborate to prevent premature initiation while the virus is not in suitable contact with a susceptible cell. This requires the existence of a trigger additional to receptor binding. For some viruses this may be a change in the environment, such as a drop in pH; for some primate lentiviruses it involves binding of a coreceptor. HIV-1 will bind to, but not enter cells expressing only its main receptor, CD4 (Maddon et al. 1986). Once the viral glycoprotein, gp120, is bound to CD4 conformational changes occur that permit it to

bind one of its coreceptors, CXCR4 or CCR5 (Alkhatib et al. 1996; Deng et al. 1996; Dragic et al. 1996; Feng et al. 1996). Once coreceptor is bound, further conformational changes and fusion occur (Sullivan et al. 1998).

1.5.2 Fusion and entry

How is the fusion process affected by whether entry is considered to be pH-dependent or (as for most retroviruses (McClure et al. 1990)) independent? Viral binding to the cognate receptor must always occur for fusion to follow. As the receptors used by virions to enter cells are there to perform some cell-specific function rather than for the benefit of the virion, the apparatus necessary for fusion is contained within the viral envelope. However, the changes that activate Env subsequent to receptor binding differ.

VSV is taken across the cell membrane by clathrin-mediated endocytosis (Sun et al. 2005) and the increasing acidity of the endosomal environment primes the viral fusion protein to initiate exit from the pathway. Entry into endosomes additionally removes the virus quickly from the harsh extracellular environment, utilising the cell's own mechanisms to aid it in crossing the plasma membrane. Clathrin-mediated internalisation is required for many other viruses (e.g. adenovirus (Meier et al. 2002), while some others can also utilise clathrin-independent pathways (e.g. influenza (Lakadamyali et al. 2004)).

Successful entry of HIV, however, generally only follows fusion at the cell surface, and is pH-independent (McClure et al. 1988). Endocytic processes usually lead to a non-productive end for HIV as the virus cannot initiate exit from the lysosomal pathway and will be degraded. This was found to be untrue in the exceptional case of human placental trophoblasts, which are polarised cells (Vidricaire et al. 2004). Infection of these cells is a key stage in mother to child transmission of HIV, and the virus may additionally be transcytosed whole across the placental cells and released intact at the other side, ready to infect nearby foetal cells (Vidricaire et al. 2004). Trophoblasts express little to none of the receptors and co-receptors for HIV, yet they can be productively infected to a moderate degree (Vidricaire and Tremblay 2005). HIV is heavily endocytosed within these cells, and cytokines released during

pregnancy may create conditions temporarily favourable for escape from endosomes. Inhibitors of endocytosis completely block infection (Vidricaire and Tremblay 2005). In this case, HIV is clearly able to exploit discrepancies in the cell biology of trophoblasts as compared to other cell lines in order to permit a-typical usage of this pathway.

Once the virus is bound by receptor and any requirements for fusion additional to receptor binding have been met, the fusion mechanism is triggered. The fusion reaction can take place at the cell surface (e.g. CD4/HIV-1) or in an endosomal compartment (e.g. Tva/ASLV) (Stein et al. 1987; Sinangil et al. 1988; Mothes et al. 2000). For the envelope of HIV-1, binding of CD4 and a coreceptor are sufficient to trigger the sequential conformational changes that eventually result in fusion. For VSV-G, a drop in pH activates the receptor for fusion. In a remarkable twist, ASLV-A Env appears to combine both strategies, using both conformational changes and a drop in pH. Firstly, Env binds to the Tva receptor, causing a conformational change to occur, but a drop in pH is necessary for full activation and completion of fusion (Barnard et al. 2006) (discussed in more detail below, in section 1.7).

The fusion reaction between viral and cellular membranes is highly energetically unfavourable, involving extensive rearrangement of the cellular membrane. The conformational changes undergone by the viral envelope proteins drive these rearrangements (Eckert and Kim 2001). Despite the difference in the conditions under which fusion is triggered, the fusion mechanisms of many viral envelope proteins is very similar. Fusion proteins can be divided into two classes: class I and class II. Class I proteins undergo a stage known as a 6-helix bundle, and class II proteins do not (Colman and Lawrence 2003; Kielian 2006). After SU binding to the cell-surface receptor, TM forms this 6-helix bundle in which it essentially bends back onto itself. The N-terminal segments form a coiled-coil and the C-terminal segments lie alongside the central coiled-coil in antiparallel configuration (Markosyan et al. 2003; Markosyan et al. 2004). Once formed, this six helix bundle is stable to well above physiologically relevant temperatures (Lu et al. 1995). The viral and cellular membranes are forced into close proximity as the fusion peptide of the viral envelope is inserted into the cellular membrane, and the membrane spanning segment is in the viral membrane (Markosyan et al. 2003). Formation of this bundle is an obligate step

and as such, is a suitable target for anti-HIV drugs. Enfuvirtide is a drug based on an anti-retroviral peptide that binds to gp41, preventing structural rearrangements (Greenberg and Cammack 2004). Class II proteins appear to show a similar approach to fusion, but with unrelated intermediate protein structures.

One of the best-understood series of binding and fusion events, supported by a wealth of structural information, is that between the receptor protein of influenza virus, haemagglutinin (HA), and sialic acids on glycoproteins and glycolipids. HA exists as a trimer of heterodimers of HA1 and HA2 (Wilson et al. 1981). Once bound to receptor, the virus will be endocytosed, and HA is dependent on the consequent drop in pH that occurs as the virus passes through the endosomal pathway to trigger a conformational change (Skehel et al. 1982; Wharton et al. 1986). A fusion peptide in HA2 is brought into close contact with, and inserted into, the target cell membrane (Carr and Kim 1993; Bullough et al. 1994), and with the other end in the viral membrane the Env-receptor complex undergoes a conformational change that pulls the outer viral and cellular membranes close into a hemi-fused intermediate. Subsequently the two progress together to full fusion, and eventually sufficient enlargement of the pore to permit the core of the virus to pass through (Skehel and Wiley 2002).

Models of retroviral Env proteins in comparison to HA appear to share elements such as potential fusion peptides, but differ in details, as would be expected from envelopes designed to bind to different receptors.

1.5.3 Reverse transcription

Once fusion has been completed, whether from the extracellular milieu or endosomes, the viral core is released into the cytoplasm and embarks on the next stage of the cycle. The core contains the viral genome, condensed by association with NC and RT both of which are essential components of the reverse transcribing core. The capsid structure enclosing the core must disassemble, as immature virions, in which the capsid core is much more stable, are non-infectious. A very limited level of reverse transcription does occur in intact virions before entry, but only increases to significant levels after core breakdown (Trono 1992; Zhang et al. 1993). Once the capsid shell

has been partly dismantled, however, components of the core do not completely disperse throughout the cell and have been tracked as bound to the proviral complex up to, and even beyond, import into the nucleus.

DNA synthesis is initiated from the 3'-OH of a specific cellular tRNA bound to the primer-binding site (pbs). A short DNA fragment is then synthesised up to the 5' end of the genome, comprising 5'R-U5-pbs. This is known as strong stop DNA, and is a useful marker for an indication of viral entry, and commencement of the earliest stage of the replicative cycle. The RNA template is then digested exo-or endo-nucleolytically as far as U5 by RNaseH, and the new DNA 'jumps' to the 3' end of the RNA template, binding the newly transcribed DNA R region to the 3' RNA R region . DNA synthesis continues, and the RNA template is concomitantly degraded with short pieces remaining as primers for plus strand DNA (Haseltine et al. 1976; Gilboa et al. 1979). During this process jumps between and within the two copies of RNA in the genome are frequent, leading to mutations and deletions or insertions. When combined with an error rate of $\sim 10^{-4}$ for the polymerase, these jumps account for the extremely high mutation rate of many retroviruses (An and Telesnitsky 2001).

A purine-rich section near the 3' end of the genome that is relatively resistant to RNAse H acts as a primer for plus strand DNA synthesis (Rattray and Champoux 1987; Rattray and Champoux 1989). The newly transcribed minus strand DNA acts as a template for synthesis from this primer. Synthesis proceeds in the 5' direction, and includes the tRNA primer still attached to the pbs at the 5' end, halting at a modified base found in the tRNA. Once the tRNA is displaced (Pullen et al. 1992), the complementary pbs sequences on the plus and minus strands pair to facilitate the second strand transfer. These steps are illustrated in fig. 1.5.3.

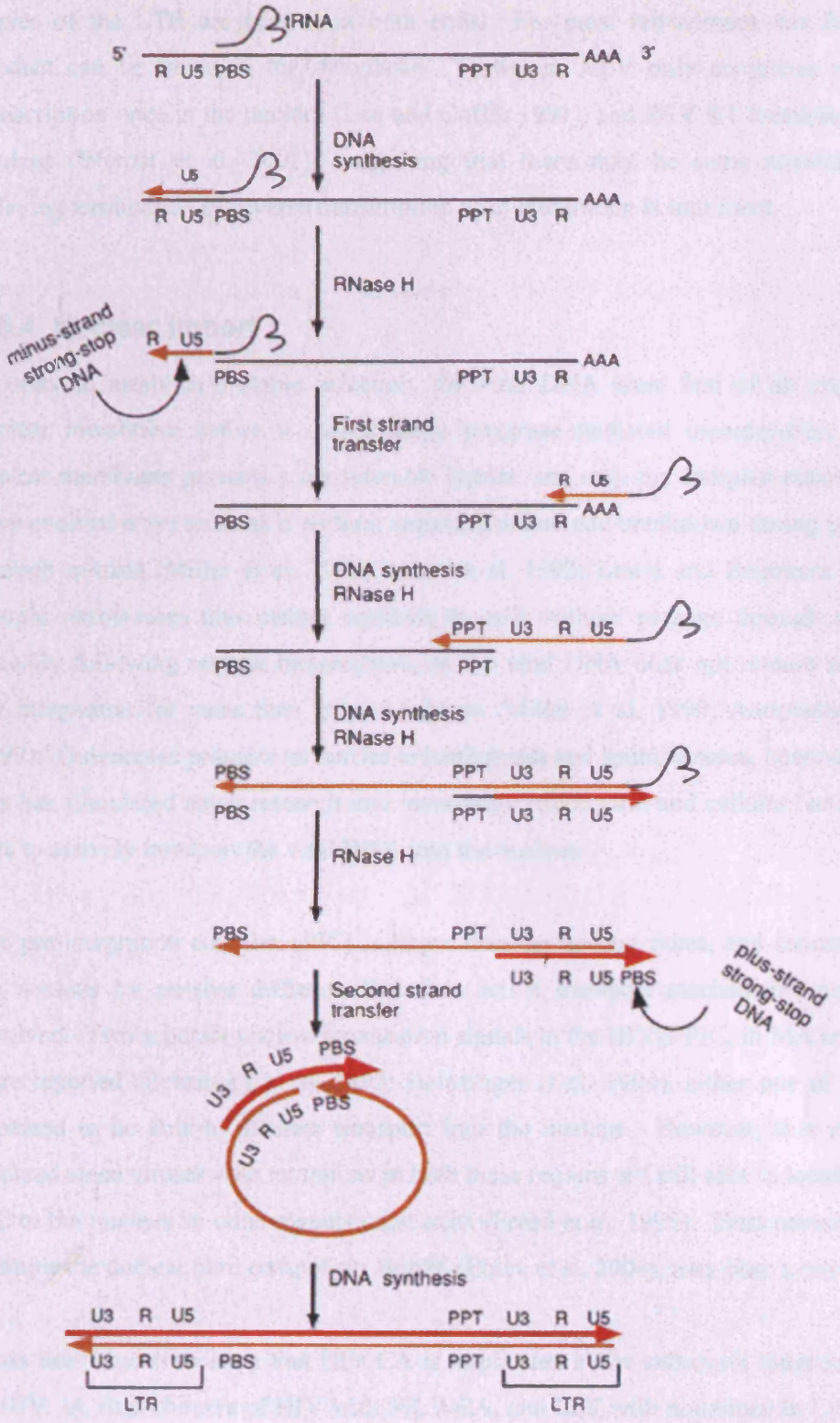


Fig. 1.5.3 Outline of the stages of reverse transcription described in the text. Taken from (Coffin et al. 1997).

The final product of RT is a double stranded DNA copy of the full genome, with copies of the LTR are present at both ends. For most retroviruses this finished product can be found in the cytoplasm. However, ALV only completes reverse transcription once in the nucleus (Lee and Coffin 1991), and RSV RT localises to the nucleus (Werner et al. 2002), suggesting that there may be some advantage to delaying termination of reverse transcription until integration is imminent.

1.5.4 Nuclear import

In order to establish a stable infection, the viral DNA must first of all cross the nuclear membrane before it can undergo integrase-mediated incorporation. The nuclear membrane presents a considerable barrier, and only the complex retroviruses have evolved ways to cross it without requiring membrane breakdown during passage through mitosis (Miller et al. 1990; Lewis et al. 1992; Lewis and Emerman 1994). Simple retroviruses thus cannot replicate in cells without passage through mitosis speedily following reverse transcription, as the viral DNA does not remain suitable for integration for more than around 6 hours (Miller et al. 1990; Andreadis et al. 1997). Quiescence presents no barrier to lentiviruses and spumaviruses, however, and this has stimulated much research into identifying which viral and cellular factors are able to actively transport the viral DNA into the nucleus.

The pre-integration complex (PIC) is larger than the nuclear pores, and cannot enter the nucleus by passive diffusion therefore active transport mechanisms must be involved. Two separate nuclear localisation signals in the HIV-1 PIC, in MA and Vpr were reported (Bukrinsky et al. 1993; Heinzinger et al. 1994), either one of which appeared to be able to mediate transport into the nucleus. However, this view is disputed since viruses with mutations in both these regions are still able to localise the PIC to the nucleus so other signals must exist (Freed et al. 1995). Host proteins, for example the nuclear pore component Nup98 (Ebina et al. 2004), may play a role.

It has been shown recently that HIV CA is implicated in the cell-cycle independence of HIV. A viral chimera of HIV with MLV CA, and HIV with mutations in CA were both unable to infect non-dividing cells (Yamashita and Emerman 2004; Yamashita et al. 2007). Markers of nuclear import, 2LTR circles, were found in the nucleus,

indicating that it is not the physical access to the nucleus that is limiting, but a step downstream of this, possibly related to retention of CA with the pre-integration complex (Yamashita et al. 2007).

1.5.5 Integration

Production of linear viral DNA alone by reverse transcription is not sufficient for founding a long-term infection; integration into the host chromosomal DNA allows the retrovirus to establish an infection on a permanent basis and is necessary for a productive infection (Englund et al. 1995). At the ends of the U3 and U5 sequences are ~10bp sequences known as *att* sites, recognised by IN (Bushman and Craigie 1990), that contain a key CA dinucleotide pair. Two terminal nucleotides adjoining this pair are removed by integrase prior to strand transfer. This CA pair thus will ultimately define the 3' ends of the integrated viral DNA, and the LTRs control of the replication of the viral genome. The second part of the reaction, the strand transfer of the vDNA into the host DNA is via an attack of the processed 3'OH ends on the target DNA (Fujiwara and Mizuuchi 1988). Integrase is the only viral protein necessary to carry out the reaction (Bushman et al. 1990; Craigie et al. 1990).

While there is no specific site in the host genome preferred for integration, the distribution of integrated provirus is not uniform. The tertiary structure of DNA appears to be more important than an extensive primary sequence in determining whether a site is preferred or not (Pryciak et al. 1992). Preferences for integration into certain areas may be retrovirus type-specific, for example, HIV appears to integrate into sites of active transcription, whereas MLV favours transcription start sites and CpG islands (Schroder et al. 2002; Wu et al. 2003; Maxfield et al. 2005). DNA in nucleosomes is targeted in preference to naked DNA, but other proteins bound to DNA (e.g. in regions where DNA is highly transcribed) may decrease access of the provirus. A 5-bp sequence, with either end on the same side of the double helix, is targeted by HIV-1 during integration (Vincent et al. 1990). This sequence is duplicated during this process, which together with the insertion of the retroviral genome explains the inherently mutagenic nature of integration.

The fact that integration is not entirely random for some retroviruses indicated that a cellular protein connected with transcription might be involved in binding IN to DNA. In 2003 lens epithelium-derived growth factor (LEDGF/p75) was found in a complex with HIV-1 IN (Cherepanov et al. 2003). LEDGF/p75 is involved in the cellular stress response, and binds both chromosomal DNA and lentiviral IN, possibly acting as a tethering factor (Maertens et al. 2003; Llano et al. 2004). Knockdown of LEDGF/p75 significantly inhibits replication of HIV (Vandekerckhove et al. 2006). When LEDGF/p75 was depleted from cells subsequently infected with HIV, integration into transcription units and genes under transcriptional control of LEDGF/p75 was less frequent, and into CpG islands was more frequent indicating that LEDGF/p75 affects the choice of target site (Ciuffi *et al.* 2005).

Although vDNA can be found in circularised forms in the nucleus, it appears that only linear DNA can be used for integration (Ellis and Bernstein 1989). Other forms found in cells are made from circularisation of the linear DNA at the LTRs, and while dead end products for the virus, are useful analytical markers of nuclear entry.

Once the viral DNA is integrated, there is little chance that it will be completely excised, although low-frequency excision of the full-length insertion by recombination between the 5' and 3' LTRs does occur. This event leaves behind a single LTR (a 'solo LTR'), and has been mostly studied for retrotransposons in yeast and Drosophila (Mager and Goodchild 1989). For germline cells, the integration of vDNA means that every past retroviral infection will leave a calling card of viral DNA in the host genome. Viruses that infect germline cells and pass down their complete genome to progeny are known as endogenous retroviruses (ERVs). ERV sequences are not essential for host cell function, and usually eventually acquire mutations that knock out viral production, leaving just the traces behind. As much as 8% of the human genome may be accounted for in this way (Smit 1996; Griffiths 2001), and the human genome project found evidence of several thousand infections by at least twenty-four different families (Griffiths 2001; Villarreal 2001).

1.5.6 Assembly and exit

Once integration is complete, the virus relies on standard host mechanisms for transcription and translation. Both spliced and unspliced mRNAs are produced, the former for production of viral proteins, and the latter to serve as genomic RNA for progeny virions. Both must be transported to the cytoplasm, and different retroviruses have evolved different ways to achieve this, as unspliced mRNAs are normally retained in the nucleus. Simple retroviruses contain *cis*-acting elements in the RNA that interact with cellular factors, or the splice site signals are relatively weak (Katz and Skalka 1990; Ogert et al. 1996). In complex retroviruses the viral Rev or Rex protein binds to their *cis*-acting elements, permitting transport without splicing (Cullen 1992). Before significant quantities of Rev are produced after infection, multiply spliced small mRNAs predominate. After the build-up of Rev to over a threshold level, singly spliced and unspliced mRNAs begin to appear in the cytoplasm (Kim et al. 1989). After this point, production of retroviral protein and assembly of progeny virions can proceed.

Gag, which is at this point uncleaved, is the only retroviral protein needed to drive the targeting of the retroviral polyprotein to the plasma membrane, and the assembly into immature particles (Freed 1998). A sequence within MA known as the M domain is the membrane-binding domain. In HIV-1, myristoylation of this domain and a cluster of basic residues together are both necessary for membrane targeting (Gottlinger et al. 1989; Zhou et al. 1994). Gag is also responsible for association of the viral genome with the nascent particle. Env proteins are synthesised into the ER membrane and trafficked to the plasma membrane, enriched at budding sites.

Interacting Gag proteins form structures that bud out from the plasma membrane, eventually pinching off as immature particles. This pinching off is inhibited by both mutations in the L domain of Gag and by cellular proteins known as tetherins. The action of tetherins can be overcome artificially by proteases, and are counteracted during replication of HIV-1 by Vpu (Neil et al. 2006; Neil et al. 2008). PR cleavage of the polyproteins occurs once the immature virion acquires a membrane via budding and release through the cell surface. Protease processing occurs, the core condenses and the newly matured virion is capable of initiating a new infection.

1.6 Vectors and Viral Pseudotypes

As retroviruses are capable of inserting genes stably into the host chromosomal DNA, they have been extensively researched and manipulated as gene delivery systems. Retroviral vectors have been developed as a way of generating replication defective virus-like particles capable of completing limited aspects of the infectious cycle of retroviruses, without the risk of associated undesirable aspects such as oncogenic potential, and the spread of the virus after an initial infection. The first stages of retroviral replication up to, and including integration, do not require any *de novo* viral protein synthesis, so genes required for generation of progeny virus, and cell exit can be excised and supplied in *trans* in the producer cell (Mann et al. 1983; Watanabe and Temin 1983). Instead, a selectable marker gene is inserted which will be translated by the cell, so that an initial round of replication can be detected (Palmer et al. 1987). A packaging cell line is one that has integrated genetic material encoding the structural proteins of the virus and the viral envelope, however, they cannot transmit or package the RNA that encodes these (Shank and Linial 1980). Alternatively, constituents of viral particles can be produced from two or more separate plasmids transiently expressed in a producer cell (e.g. by transfection), encoding *gag-pol,* and the viral envelope. A third plasmid encoding a gene of interest, and potentially a selectable marker can be co-transfected. This must contain a ψ sequence between the promoter and the inserted gene to direct uptake and packaging of the RNA into virions, and *cis*-acting sequences that direct stages of reverse transcription and integration (Soneoka et al. 1995; McBride et al. 1997). All packaged vectors used to transfer genes in this study used selectable markers driven from an IRES (internal ribosome entry site) to assay for gene expression (Jang et al. 1990). Production of the gene of interest is driven from the 5' promoter sequence, and production of the selectable marker from an IRES located between the gene of interest and the selectable marker. Protein production from the IRES can be reasonably assumed to guarantee production of the gene of interest. Selectable markers used in this way include fluorescent markers (e.g. YFP), and neomycin resistance (see fig. 2.3.1 for schematic representations of vectors)

An advantage of using separate gag-pol and env components to generate virus particles is that component parts do not need to come from the same virus (Zavada 1972). That is, MLV gag-pol can be transfected along with env plasmid from HIV-1, ASLV-A or, as is most widely used, VSV (Yee et al. 1994). The particles formed will have the structural proteins encoded from the MLV *gag-pol*, and envelope from a separate (retro)virus. This is known as pseudotyping, and is widely used to introduce virions to a variety of cell types not normally permissive to infection mediated by the cognate envelope protein. An additional benefit is that the chance of recombination to generate replication competent virus, a key safety consideration, is reduced.

1.7 Entry of ASLV-A

A great deal of the work presented here includes a viral envelope and receptors of an alpharetrovirus, ASLV. Infection by ASLV causes B-cell lymphomas. There are currently 10 groups of ASLVs, A-J, designated according to host range, receptor subgroups and interference, and neutralisation by antibodies (Weiss 1993). Groups A-E can be further grouped according to whether they are non-cytopathic (A, C and E) or cytopathic (B and D) (Weller and Temin 1981). Three separate loci are responsible for controlling susceptibility to these five groups: *tva*, *tvb* and *tvc* (tv, tumour virus). Tva and Tvc permit entry of groups A and C, respectively, and two alleles of *tvb* permit entry to either B, D and E, or just B and D (Payne and Biggs 1964; Crittenden et al. 1967; Duff and Vogt 1969). *Tva* and *tvc* are genetically linked (Payne and Pani 1971). The normal functions of Tva, Tvb and Tvc are not known.

1.7.1 Tva800 and Tva950

The receptors for ASLV-A were identified by examining chicken genomic DNA that rendered African green monkey COS-7 cells susceptible to infection (Young et al. 1993). Further characterisation required examination of the quail homologue, as the chicken gene was not transcribed at a detectable level, and many ORFs were encoded. Two alternatively spliced genes were identified from quail, both of which encoded proteins containing identical 83 amino acid sections and included an extracellular receptor domain with an LDL-A (low density lipoprotein) motif. This motif was first found in the LDL-receptor, is approximately 40-amino acid long including seven

ligand-binding repeats (~50% identical) and is also found in many other cellular proteins (Tolleshaug *et al.* 1982; Herz 2001). The difference between the two arose in their attachment to the membrane, by either a single membrane spanning region, or a glycosylphosphatidylinositol (GPI) anchor (Bates et al. 1993). These were named Tva950 (transmembrane linked protein) and Tva800 (GPI anchor). Both comprise an N-terminal signal sequence (1-19) and the extracellular domain (20-102). Tva950 then consists of a transmembrane domain (103-125) and a small cytoplasmic tail. Tva800 has a C-terminal GPI anchor signal sequence: it is synthesised into the ER like a transmembrane protein, but once completed a carboxy terminal signal directs GPI-anchor attachment (Doering et al. 1990; Moran and Caras 1991). GPI anchors share a common phosphoethanolamine-Mannose$_3$-phosphatidylinositol core, but potential modifications of side chains lead to a high level of heterogeneity. A schematic of Tva800 and Tva950 is shown below in figure 1.7.1.

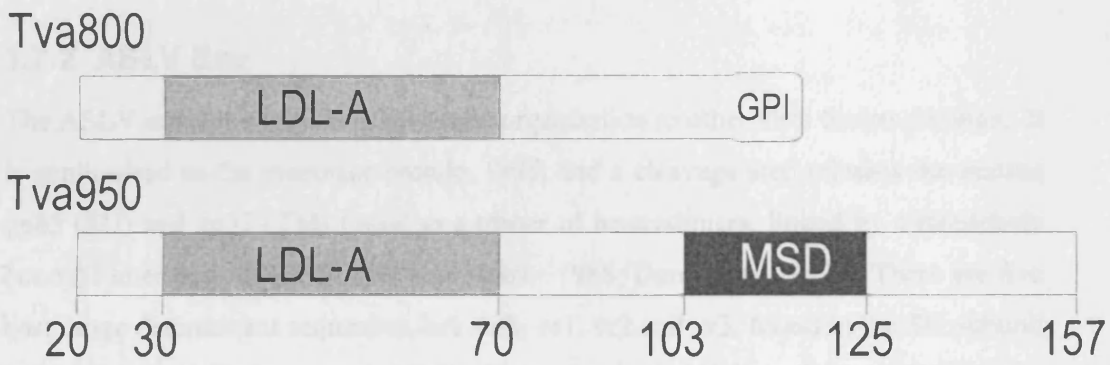


Figure 1.7.1 Simplified outline of the Tva800 and Tva950 proteins. The two are identical up to amino acid 103, after which Tva800 is modified to include a GPI anchor, whereas Tva950 has a single membrane-spanning domain.

The LDL-A motif makes up the bulk of the extracellular receptor structure at 47 residues, and is sufficient to mediate viral binding (Rong and Bates 1995; Damico et al. 1998). Calcium is required for correct folding (Wang et al. 2001), and the tertiary structure is also held in shape by three pairs of disulphide bonds between six invariable cysteine residues (Belanger et al. 1995). The structure of this part of Tva was determined in solution by NMR spectroscopy (Tonelli *et al.* 2001; Wang *et al.* 2002a), which has aided elucidation of the precise roles of specific amino acids identified as key determinants of viral binding, although the two final structures differed in interpretation at the N-termini. The key differences between Tva LDL-A

and other LDL-A structures are concentrated at the N-terminus, and appear to arise from extra amino acids in loops of Tva. H38 and W48 were identified as critical viral binding or entry residues (Zingler and Young 1996; Rong *et al.* 1998a; Rong *et al.* 1998b; Wang *et al.* 2002b), and in the solution structures lie at the exposed surface of the molecule, available to interact with the viral envelope. Interestingly, they are not conserved amongst the LDL-A family and so must be essential for viral binding or fusion events, rather than correct receptor folding. Other key residues identified are buried deep within the molecule, and so are more likely to be important in folding or maintaining the structure (Tonelli *et al.* 2001; Wang *et al.* 2002a). A hydrophobic patch on the surface of Tva has been suggested as a point of contact with the envelope protein, or as involved in correctly orientating the interaction. The natural function of Tva is not known, so it is currently impossible to compare the binding of ASLV Env with the physiological ligand to Tva.

1.7.2 ASLV Env

The ASLV envelope protein is similar in organisation to other viral fusion proteins. It is synthesised as the precursor protein, Pr95, and a cleavage step releases the mature gp85 (SU) and gp37 (TM) found as a trimer of heterodimers, linked by a disulphide bond (Hunter et al. 1983; Einfeld and Hunter 1988; Dong et al. 1992). There are five host range determinant sequences, hr1, hr2, vr1, vr2 and vr3, found in the SU subunit (Dorner et al. 1985; Bova et al. 1988). There are several N-linked glycosylation sites in SU, but none are in regions that form hr1 or hr2 which suggests that oligosaccharides are not involved in receptor recognition, although they may be required for correct overall folding of the protein, and therefore indirectly be determinants for receptor binding (Delos et al. 2002).

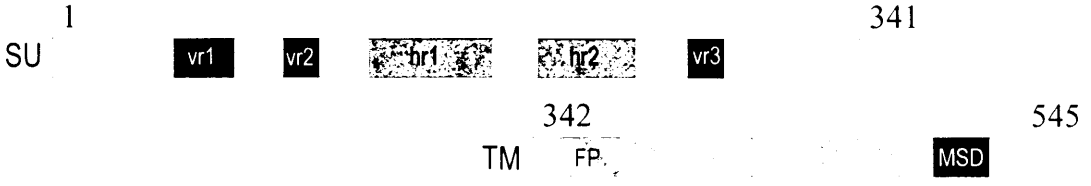


Fig 1.7.2 A representation of the structure of ASLV Env. The two parts are generated from cleavage of a single polypeptide, and are linked by a disulphide bond, represented by a dashed line. The hashed domains represent heptad repeats. SU – surface protein, TM – transmembrane, vr – variable region, hr – host range, FP – fusion peptide, MSD – membrane spanning domain. Simplified from (Barnard and Young 2003).

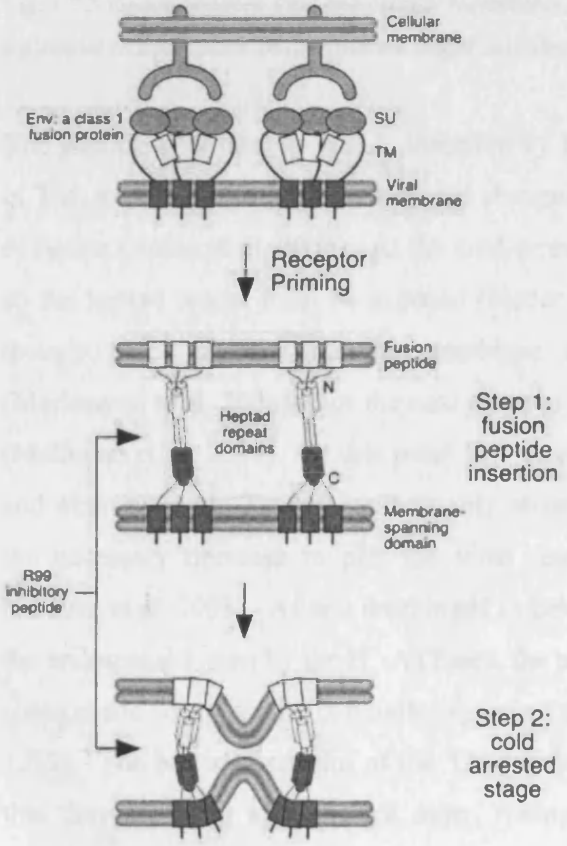
Viruses that have been selected for their ability to infect a wide range of host cells carry mutations in the hr regions (Taplitz and Coffin 1997), and mixing and matching sequences from these regions alone can have a significant effect on host permissivity of viruses. For example, insertion of the hr2 sequence from ASLV-E Env into ASLV-B Env permits a virus to infect both ASLV-E and ASLV-B receptor-expressing cells (Tsichlis et al. 1980; Dorner et al. 1985). Host range is not as affected by alterations in vr1 and vr2, but vr3 may act as a supplementary determinant to hr sequences in some cases; the reason for the high level of variability in vr1 and vr2 is not clear.

Insertion of a fusion peptide into the target cell membrane by the activated viral envelope protein is a step common to enveloped viruses during entry. During entry mediated by HA (Harter et al. 1989), HIV-1 (Gordon et al. 1992) and SIV (Horth et al. 1991), the fusion peptide likely forms an amphipathic helix which exposes its hydrophobic residues to the lipid bilayer. The likely fusion peptide in ASLV Env is found in the N-terminus of TM and like other viral fusion peptides, has a proline near the centre. Mutations of this proline do not abolish inter-Env interactions, but do weaken them, and prevent ASLV entry.

1.7.3 Mechanism of entry via ASLV-A Env

Despite apparently contradictory data in recent years pertaining to the mechanism of ASLV-A fusion and entry, a general outline of sequential steps that must occur during entry now seems clear. Uniquely ASLV is not easily classified as using either a pH-dependent or pH-independent route of entry, but borrows from both mechanisms. The envelope proteins of viruses that are pH-dependent are usually activated by low pH, as found in endosomes (e.g. influenza HA). Exposure of such viruses to low pH in a cell-free environment results in premature triggering of the fusion mechanisms, rendering the virus inactive (Stegmann et al. 1987). Similarly, exposure of cells to lysosomotropic agents that prevent acidification of the endosomes causes the virus to be blocked, trapped within the endocytic pathway, unable to initiate fusion. Viruses that are pH-independent (e.g. HIV-1) initiate fusion at the cell surface, and are not affected by changes in pH. Changes in Env structure that bring about fusion in this latter group are initiated solely by receptor (and often co-receptor) binding (White 1990; Weiss 1993).

Entry of ASLV-A into cells can be blocked by the addition of lysosomotropic agents, and further exposure of cells to low pH overcomes this block, suggesting that a low pH step is necessary during entry (Mothes et al. 2000). However, ASLV-A virions are not inactivated by low pH (Gilbert et al. 1990; Mothes et al. 2000), and lipid mixing appears to occur at neutral pH (Gilbert et al. 1990; Hernandez et al. 1997). The entry mechanism of ASLV-A maps to the envelope protein alone, as pseudotyping ASLV-A cores with ecotropic MLV Env, which mediates pH-independent entry, abolishes the sensitivity to lysosomotropic agents (Mothes et al. 2000). In order to tie these data together, a mechanism of entry was proposed which involves a unique two-stage process (Mothes et al. 2000). First of all, ASLV-A Env binds to Tva at the cell surface, at neutral pH. The receptor-Env complex then proceeds to an extremely stable stage that can be paused at 4°C. By binding to the receptor, Env is primed for fusion, but will not proceed without a drop in pH. These stages are illustrated in steps 1 and 2 of figure 1.7.3.



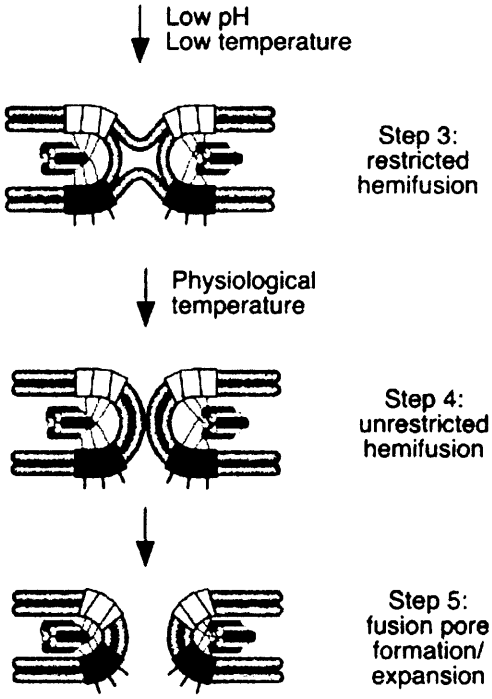


Fig. 1.7.3 Fusion between viral and cellular membranes, mediated by ASLV Env binding to receptor – a pictorial representation of the different stages described in the text. From (Barnard et al. 2006).

The peptide R99 inhibits ASLV infection by binding to an N-terminal heptad repeat in TM, exposed during conformational changes, and has been used to track the stages of fusion (Netter et al. 2004). At the cold-arrested stage, infection is inhibited by R99 so the heptad repeat must be exposed (Netter et al. 2004), and the fusion peptide is thought to be inserted into the membrane although no lipid mixing takes place (Markosyan et al. 2004). For the next stage to occur, Env must be exposed to low pH (Melikyan et al. 2004). At this point Env is very firmly inserted into the membrane, and when bound to Tva800 can be stably arrested here for over 6 hours by preventing the necessary decrease in pH; the virus remains infectious (Mothes et al. 2000; Narayan et al. 2003). After a drop in pH to below 5.5 (due to pumping of protons into the endosomal lumen by the H^+-ATPase), the primed Env undergoes a conformational change, and forms a 6-helix bundle typical of class I viral fusion proteins (see section 1.5.2). The N and C termini of the TM segment of Env come into closer contact so that they are lying against each other, resistant to SDS, and the viral and cellular membranes are forced into closer proximity with each other. At 4°C restricted fusion occurs between the outer viral and cellular lipid layers but no pore is formed. This

step can be tracked visually by the disappearance of a fluorescent lipid label from the virus membrane (Melikyan *et al.* 2005). At 37°C, fusion occurs, and a pore between the virus and the cell is gradually widened (Melikyan et al. 2005).

1.8 Inhibition of Viral Replication

Viruses are parasitic, and infection of the host can be either commensalistic or fatal. Of the latter, virus-induced diseases may be highly pathogenic and directly affect the host within hours, for example. influenza, or indirectly over many years, as in HIV-induced AIDS (Villarreal et al. 2000). Naturally, host organisms have evolved anti-viral strategies, and the immune system can produce a whole range of defences at the molecular level to fend off infections. Successful viruses have, of course, frequently found ways of efficiently circumventing these defences. In studying these defence mechanisms and the corresponding viral evasions much can be learnt about both cellular and viral functions.

1.8.1 Fv susceptibility genes

Some of the various intracellular defences were first described decades ago. In 1957 a cell-free extract was described, capable of inducing erythroleukaemia in adult mice (Friend 1957a; Friend 1957b). The aetiological root of this disease, Friend virus, is a complex of two retroviruses, one of which is the helper virus Fr-MLV (Kabat 1989; Ben-David and Bernstein 1991) and the other the pathogenic but replication defective spleen forming focus virus, which together cause leukaemia in spleens of mice. Studying resistance to Friend virus has identified several genes that confer resistance to the host from pathogenesis in several different ways. Friend virus susceptibility 1 (*Fv1*) was first described in the 70s (Lilly 1970), and cloned in the 90s (Best et al. 1996), but the mechanism of action remains unknown. Fv2 (or truncated Stk) (Axelrad 1969; Persons et al. 1999) regulates cycling in early erythroid progenitors, and also blocks proliferation of Friend virus-infected cells (Persons et al. 1999). Cloning of the *Fv4* locus revealed a defective endogenous provirus that encodes a fragment of envelope protein. When this is expressed, it binds to and downregulates the receptor, so that incoming virus cannot bind and the cells are rendered resistant. Fv1 and Fv4 do not interact with cellular components, but block infection in isolation.

Cloning of *Fv1* (Best et al. 1996) revealed a Gag-like protein, with sequence similarity to a family of endogenous retroviruses called HERV-L (Benit et al. 1997). Inheritance of resistance is dependent on a single locus, and so far has not been shown to be dependent on any other cellular factor. There are at least two alleles that provide resistance to certain strains of MLV (Stevens et al. 2004). $Fv1^b$ (from Balb/c mice) confers resistance to N-tropic MLV, and $Fv1^n$ (from NIH 3T3 mice) resistance to B-tropic MLV. A third type of MLV, NB-tropic (or Moloney MLV) is able to infect both. The primary determinant of restriction is found in the viral *gag* gene at position 110 within CA, but determinants of NB-tropism are also found elsewhere within CA (DesGroseillers and Jolicoeur 1983; Boone *et al.* 1988). B-tropic MLV has a glutamate at this site, and N-tropic MLV an arginine. Restricted viruses that enter Fv1-positive cells are blocked post-reverse transcription (Jolicoeur 1979; Pryciak and Varmus 1992), but despite a wealth of genetic data and some structural and biochemical data on CA (Mortuza et al. 2004) and Fv1 (Bishop et al. 2006), the mechanism and timing by which this occurs remains unclear. CA must be properly processed (Dodding et al. 2005) and within the context of a pre-integration complex, but direct binding of Fv1 and CA has not yet been conclusively demonstrated. It could occur as soon as immediately after entry, so that the virus is effectively doomed despite continuing to synthesise products of reverse transcription.

1.8.2 Trim5α

Pseudotyping MLV cores with VSV-G allows a range of host cells other than murine cells to be infected. A block to N-tropic MLV infection in human cells that was similar to Fv1 was seen, despite there being no homologue of Fv1 in human cells, and was labelled Ref1 (Restriction factor 1) (Best et al. 1996; Towers et al. 2000). Restriction of HIV-1 in some nonhuman primates was called Lv1 (lentiviral susceptibility factor 1) (Cowan et al. 2002; Munk et al. 2002). Lv1 and Ref1 were later shown to be species-specific variants of a single protein, Trim5α (Keckesova et al. 2004; Stremlau et al. 2004; Yap et al. 2004). Since this time, several other blocks have been described (Schmitz et al. 2004; Cutino-Moguel and Fassati 2006; Pineda et al. 2007).

Many similarities exist between phenotypes exhibited by Trim5α- and Fv1-positive cells when infected by restricted virus. The first clue that both were due to a dominant factor expressed in restrictive cells was their saturable nature. Fv1 restriction in cells can be overcome at very high titres of restricted virus (Hartley et al. 1970). Alternatively, cells expressing either Fv1 or Trim5α can be pre-treated with restricted virus at high MOI. A second round of infection with a low MOI of restricted virus will give high titres, indicating that the first round of infection has saturated all of the pool of the restricting factor in the cell. This is known as abrogation (Duran-Troise et al. 1977; Bassin et al. 1978; Boone et al. 1990; Towers et al. 2002). When restrictive and permissive cells were fused, infection of these heterokaryons was found to result in titration curves similar to the restricted cells alone, which indicates that restriction is not due to absence of a cellular factor produced by permissive cells, but due to the presence of a negative factor in restrictive cells (Tennant et al. 1974; Cowan et al. 2002).

Trim5α was identified from a screen of rhesus monkey cDNA, which identified a gene that when introduced into permissive cells rendered them restrictive to HIV-1 replication (Stremlau et al. 2004). It is a member of the tripartite motif family of proteins, and contains RING (really interesting new gene), B-box, coiled-coil and B30.2 (or SPRY) domains (Reymond et al. 2001). The B30.2 contains the determinants for Trim5α specificity (Yap et al. 2005), and chimeras made of CA-binding proteins in place of the B30.2 domain fused to the RBCC also function as restriction factors, suggesting that it may be the overall structure of the RBCC that mediates restriction (Nisole et al. 2004; Sayah et al. 2004; Yap et al. 2006). Trim5α blocks restricted virus at an earlier stage than Fv1, as no products of reverse transcription are made. The mechanism of Trim5α mediated restriction remains controversial, but studies of the structure and mutational studies have shed light on aspects of the protein essential for function (Stremlau et al. 2005; Javanbakht et al. 2006; Ohkura et al. 2006). In owl monkeys, the B30.2 has been replaced by cyclophilin A (CypA) (Nisole et al. 2004; Sayah et al. 2004), but the rest of the RBCC remains unaltered. This Trim5-CypA remains able to restrict HIV-1, but not HIV-1 G89V, which has a mutation in the CypA binding site. This implies that the B30.2 region contains crucial determinants for CA binding, and the RBCC mediates the mechanism of restriction.

Current models of the mechanism of restriction suggest that Trim5α binds to the incoming core and either accelerates the uncoating with a rapidity that renders the core unviable (Stremlau *et al.* 2006a), or causes sequestration and degradation by the proteasome (Stremlau *et al.* 2004; Anderson *et al.* 2006; Campbell *et al.* 2007a). Curiously, blocking the proteasome with MG132 permits the virus to proceed with reverse transcription, even though ultimately it remains restricted (Anderson et al. 2006). A similar shift in timing was also seen when artificial Trim-cyclophilin fusion proteins were made from fusion of CypA to either the end of the coiled-coil (short), or in place of the B30.2 (long) of African green monkey Trims 1, 18 and 19 (Yap et al. 2006). All chimeras restricted HIV-1 replication, but assessment of the timing revealed that the 'short' versions of Trims 1 and 18 fusions permitted reverse transcription to be completed and nuclear entry to take place, whereas the 'long' blocked pre-reverse transcription. These studies have shown that binding of restriction factor does not necessarily presuppose immediate cessation of the viral life cycle and also that the mechanism of restriction probably involves a combination of events, all of which are mediated by the same RBCC domains.

1.8.3 APOBEC3G

In the 1990s it was noted that the requirement for the HIV accessory gene vif was producer cell dependent, and that the replication of progeny virions was affected by whether vif was present in the parent virion. A cDNA subtraction strategy was used to identify genes expressed in a non-permissive cell line compared to a permissive one (Sheehy et al. 2002). The factor Apolipoprotein B mRNA editing enzyme, catalytic polypeptidelike 3G (APOBEC3G) was identified, which in the absence of Vif is incorporated into new virions. In the next infection APOBEC3G converts cytosines to uracils during minus-strand DNA synthesis (Yu et al. 2004). Up to 20% of minus-strand can be hypermutated in this way, and the destabilisation can lead to degradation of the new viral DNA. APOBECs are members of DNA cytosine deaminases, involved in mRNA editing and immunoglobulin gene diversification. Vif induces APOBEC3G degradation through the ubiquitin-proteasome pathway (Yu et al. 2003; Mehle et al. 2004). Other APOBECs are also found in a cluster of 5-7 APOBEC3 genes in primates at a single locus on chromosome 22 (Jarmuz et al. 2002). APOBEC3 A, B, C, F, G, and D and E are all known, but D and E don't seem

to give rise to any protein product. When originally described these were all just classed as cytidine deaminases (Jarmuz et al. 2002), and the studies that lead to revelation of the anti-HIV-1 activity were entirely separate (Sheehy et al. 2002).

1.8.4 Lv2

In 2001 it was reported that some primary isolates of HIV-2 could bind and enter, but not replicate in certain human cell lines (McKnight et al. 2001). These HIV-2 isolates were samples from The Gambia (Schulz et al. 1990), and predominantly used the coreceptor CXCR4 (McKnight et al. 1998). It was noted that expression of the correct receptor/coreceptor combination, however, was not always sufficient to ensure successful viral replication, suggesting that other factor(s) influenced viral replication (McKnight et al. 1998). The cells in question were human cells (HeLa and U87*) engineered to express CD4. U87* cells were also made to express CXCR4 (Clapham et al. 1991; Deng et al. 1996), which HeLa cells express at a low level endogenously (McKnight et al. 1997). Isolate virus could reportedly enter and reverse transcribe in HeLa CD4 cells but progressed no further in the infectious cycle, but could complete in U87* cells. However, isolate cores pseudotyped with VSV-G were able to infect fully both cell types (McKnight et al. 2001).

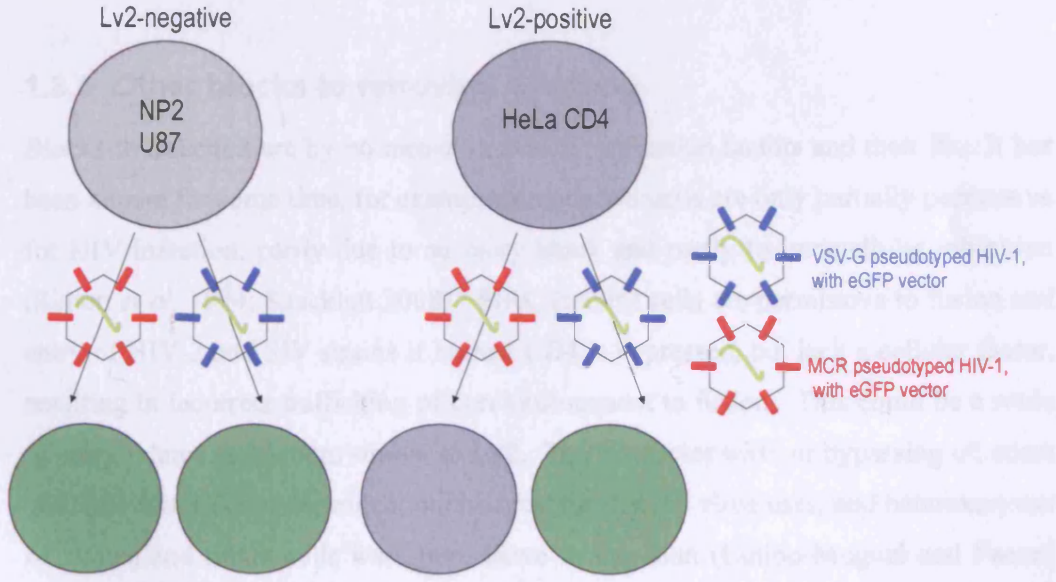


Fig. 1.8.4 A pictorial representation of the basic premise behind restriction by Lv2. HeLa CD4 cells are resistant to infection by MCR pseudotyped, GFP-encoding vectors (red envelopes) but can be infected by VSV-G pseudotypes (blue envelopes), detectable by eGFP expression in the cells upon successful infection (green cells). NP2* and U87* cells are susceptible to infection by both pseudotypes.

A molecular clone of the virus restricted on HeLa CD4 cells was constructed and analysed, and compared to a clone of a virus that was able to replicate in an unrestricted manner on both HeLa CD4 and U87* cells. The two were dubbed MCR (molecular clone restricted, the primary isolate) and MCN (molecular clone non-restricted, the T-cell line adapted isolate) respectively (Schmitz et al. 2004). The difference in replicative potential was mapped to key mutations in the *gag* (specifically *CA*) and *env* genes. It was postulated that usage of MCR Env or MCN Env delivered the virus into the cell by one of two different routes, potentially delivering the virus into a cellular compartment where an anti-viral factor was able to annul the infection. The CA sequence of the virus determines whether once in this intracellular compartment the virus will be susceptible to the anti-viral factor or not, hence Gag is also a determinant of restriction. It was concluded that a restriction factor-like activity was at work, as the determinant of Fv1 and Trim5α restriction is CA. However, this also represented a novel restriction as no previous route of entry component had been reported for other restrictions, and as such, it was named Lv2. A subsequent paper confirmed the entry dependency, and classified the pathway that led to Lv2 restriction as being lipid raft-dependent, pH independent and requiring membrane cholesterol (Marchant et al. 2005). So far, the putative factor(s) or conditions for Lv2 restriction have not been cloned or further described.

1.8.5 Other blocks to retroviral infection

Blocks to infection are by no means limited to restriction factors and their ilk. It has been known for some time, for example, that rodent cells are only partially permissive for HIV infection, partly due to an entry block and partly to intracellular inhibition (Simon *et al.* 1994; Shacklett 2008). SIRC (rabbit) cells are permissive to fusion and entry of HIV-2 and SIV strains if human CD4 is expressed, but lack a cellular factor, resulting in incorrect trafficking of cores subsequent to fusion. This could be a route of entry-related restriction, similar to Lv2. The encounter with, or bypassing of, cores with this factor is not dependent on the receptor that the virus uses, and heterokaryons of human and rabbit cells were permissive to infection (Cutino-Moguel and Fassati 2006).

Studies to uncover novel restriction factors have frequently used gene libraries as a way of introducing high-level expression of exogenous genes into a permissive cell-type. Genes that render the permissive cells non-permissive to further infection are isolated, and the inserted gene characterised. This is how Trim5α was identified (Stremlau et al. 2004), and how a zinc-finger protein ZAP (zinc-finger anti-viral protein) was discovered (Gao et al. 2002). Expression of ZAP specifically causes loss of cytoplasmic viral mRNAs, while nuclear mRNAs are unaffected.

In a slightly different approach, cells permissive to VSV-G pseudotyped MLV vector were chemically mutagenised and those that were subsequently non-permissive to infection were enriched by cell sorting. Cells were further engineered to express Tva800 and those that were non-permissive to ASLV-A Env-pseudotyped MLV, but permissive to ASLV-A vector were characterised (Bruce et al. 2005). A cell line with over 10-fold difference in permissivity to MLV vector compared to ASLV-A vector was selected. Expression of another zinc-finger protein, the transcription factor ZASC-1 was identified as being disrupted in this cell line. This protein was shown to act during or after integration, and transcription was reduced in ZASC-1 negative cell lines. The selective effects against MLV but not ASLV arise as ZASC-1 can bind to MLV U3, but not the ASLV U3 region (Bruce et al. 2007).

1.9 Other Factors Influencing Viral Entry

Uncovering the breadth of mechanisms by which viruses can enter cells has proceeded hand in hand with progress in understanding cellular pathways. Surprisingly few appear to be able to initiate productive infection at the cell surface. Most have deliberately evolved to use cellular uptake machinery. These mechanisms enable them to bypass the plasma membrane and to be delivered into the cytosol from further down the endocytic pathway as required. The number of studies in the areas of intracellular trafficking and the different pathways of viral entry is huge, and routes differ not only between different virus families, but also within families, and even for the same virus in different cell types. A summary of the main pathways of cellular uptake of external entities as will generally relate to receptors and viruses used in these studies is presented, and how different virus families use these pathways.

As the virus passes through different cellular compartments, different challenges present themselves according to the nature of those compartments. Viruses that pass through endosomes must be resistant to low pH, whereas viruses that fuse at the cell surface must find ways to bind and initiate fusion in a potentially harsh extracellular environment. Viruses that have evolved to use one route may not be able to use another if this route is not available, either because they cannot overcome the physical barriers to replication or they encounter a biological agent that they have not evolved resistance to. Viruses can be rerouted down different pathways by pseudotyping the cores with different envelope proteins (see section 1.6). Experiments using viral cores pseudotyped in this way can alter not just the route that the virus takes, but also the timing of the cycle, and potentially also the state in which the virus enters the cytoplasm.

1.9.1 Endocytosis

Selective endocytosis is usually initiated by binding of the virus to its cognate receptor. One of several distinct types of events leading to uptake can then occur. A major uptake pathway is mediated by clathrin (Royle 2006; Pauly and Drubin 2007; Ungewickell and Hinrichsen 2007). Clathrin is a molecule that forms a coat around pits in the plasma membrane, resulting in deformation and eventually vesicle scission (Heuser 1980; Edeling et al. 2006). Some pre-assembled pits exist at the plasma membrane, but typically a clathrin lattice will be assembled around membrane pits in response to an internalisation signal in the cytoplasmic tail of the receptor. There are at least four types of internalisation signals identified (Traub and Lukacs 2007), recognised by adaptor proteins, which include AP-2, epsin, Eps15, and most of these adaptor proteins can bind to clathrin and to each other (Ohno 2006; Ungewickell and Hinrichsen 2007).

The assemblage then invaginates, and pinches off from the membrane (Merrifield et al. 2005). Once entirely internalised, the clathrin coat disassembles and the vesicle fuses with the endosomal pathway, delivering the contents to an early endosome which progresses to a late endosome, with commensurate changes in the lumenal pH accordingly (Edeling et al. 2006). Viruses that enter via clathrin-mediated endocytosis either exclusively or optionally are legion, including Semliki Forest virus

(Helenius et al. 1980), VSV (Sun et al. 2005), equine infectious anaemia virus (Brindley and Maury 2008), Rice dwarf virus (a reovirus) (Wei et al. 2007), and influenza (Sieczkarski and Whittaker 2002b; Lakadamyali *et al.* 2004). These viruses usually have a fusion mechanism triggered by a drop in pH, which once reached in the acidifying endosome will result in viral exit into the cytosol. Different viral fusion proteins are triggered by different pHs, for example, SFV requires a pH of less than 6.2 (White and Helenius 1980; Marsh and Helenius 1989), and is not affected by inhibitors of late endosome function (Sieczkarski and Whittaker 2003), indicating that the site of fusion out of the endocytic pathway is at the early endosome. Tracking of influenza virus with conformation specific antibodies showed that haemagglutinin was not competent for fusion until it reached the late endosome, where the pH is typically closer to 5 (Sieczkarski and Whittaker 2003). VSV has a wider range for endocytosis of between 5.5-6.3, and so will fuse successfully somewhere between the two (White et al. 1981; Matlin et al. 1982; Marsh and Helenius 1989). Fusion can be induced at the plasma membrane by incubating cells in an acidified medium, but this does not always result in a productive infection, as the viral Env may be prematurely triggered while not in contact with the cell membrane. Other pathogens, such as Listeria, also use clathrin and clathrin-associated rearrangements of the cell membrane to enter cells (Veiga et al. 2007).

Clathrin-independent forms of endocytosis can be broadly grouped into two categories; those that are dynamin-dependent and those that are dynamin-independent (Mayor and Pagano 2007). Caveolar endocytosis is a clathrin-independent, dynamin-dependent mechanism of endocytosis that bypasses the acidifying endosomal system, and caveolae are typically formed from lipid raft areas of the plasma membrane, where receptors for molecules that are taken up by caveolae are found (Conner and Schmid 2003; Nichols 2003; Lajoie and Nabi 2007). A constituent component of caveolae is caveolin-1, which binds strongly to cholesterol and is highly resistant to extraction by detergent, emphasising the strong link between caveolae and lipid rafts (Sargiacomo et al. 1993). Internalisation is slower than clathrin-mediated endocytosis, non-constitutive (Thomsen et al. 2002; Conner and Schmid 2003), and directs the incoming virions to caveosomes. These represent a class of internal transport vesicles that are separate from endosomes (Nichols 2003). This distinction is not trivial and could have implications in pathogenesis, for example, the chemokine

receptors CCR5 and CXCR4 that function as co-receptors for HIV are endocytosed via caveosomes and clathrin-mediated uptake respectively (Venkatesan et al. 2003). The virus classically studied in association with caveolae is SV40. The virus has been visualised during binding to small pockets at the cell membrane, caveolin-1 recruitment, and virus internalisation (Anderson et al. 1996), and subsequent trafficking along microtubules to the ER. Other viruses that use caveolae for entry include BK virus (a polyomavirus) (Moriyama et al. 2007), and respiratory syncytial virus may use internalisation by caveolae for transport directly to the ER (Shin and Abraham 2001).

A separate form of extracellular uptake is macropinocytosis, which occurs when membrane protuberances enclose around a volume of liquid, that then becomes a vacuole (Conner and Schmid 2003). This is a non-specific form of entry, which is unlikely to be chosen as a favoured route by viruses. Vaccinia virus may be an exception. This is a large virus, that can have one of four combinations of coats, and in a curious twist to the uncoating process, appears to lose some of these by dissolution at the surface without fusion, after which the virus enters by macropinocytosis, which could be virally stimulated (Law et al. 2006; Dodding 2008).

1.9.2 Rab proteins

For those virions that enter via the endocytic routes, intracellular travel may be facilitated by Rab (Ras-related in brain) proteins. These are small guanosine triphosphatases, members of the wider Ras superfamily, found at characteristic sites in the trafficking pathways, controlling fusion and docking of vesicles. There are over 70 Rab and Rab-like members found (Schwartz et al. 2007). They cycle between GDP-bound inactive and GTP-bound active states, and conversion between the two states is stimulated by guanine nucleotide exchange factors (Goody et al. 2005). Rab proteins are found in almost all intracellular membranes and the plasma membrane (Schwartz et al. 2007). The most studied function of Rab proteins is their role in controlling intracellular traffic. Rabs regulate endocytic and secretory routes, anterograde and retrograde trafficking, and also organise the membranes of subcellular organelles into microdomains, ready for subsequent trafficking to different destinations (Barbero et al. 2002; Vonderheit and Helenius 2005; Grosshans et al.

2006). They play specific roles in different types of cells, for example, in generation of polarity in epithelial cells by regulating junctions, and maintaining directional flows in transport circuits to cilia (Schwartz et al. 2007).

Within the cell, Rabs are characteristically found associated with a specific population of sub-cellular organelles, and pathways can be distinguished according to the presence or absence of a particular Rab regulating them. For example, Rab5 is found on early endosomes, is important for sequestering ligands into clathrin-coated pits, and also regulates early endosome-early endosome fusion (Gorvel et al. 1991; McLauchlan et al. 1998; Barbieri et al. 2000; Pelkmans et al. 2004). Rab7 is found on late endosomes, and is important for regulation of early-late endosomal traffic (Feng et al. 1995; Vonderheit and Helenius 2005). Rabs 4 and 11 are involved in recycling of materials to the cell surface, and Rab 9 aids transport between late endosomes and the Golgi (van der Sluijs *et al.* 1992a; van der Sluijs *et al.* 1992b; Lombardi *et al.* 1993; Khvotchev *et al.* 2003; Ganley *et al.* 2004).

Dominant negative (DN) forms of these proteins have been important in elucidating function (Feig 1999). These usually have a mutation in the active site and are unable to bind GTP, and when overexpressed in a cell can down-regulate certain pathways, although without completely blocking them. For example, using Rab5DN and Rab7DN it was determined that Rab5, but not Rab7 is required for entry of dengue virus and West Nile virus (Krishnan et al. 2007), but entry of influenza virus is inhibited by down-regulation of both (Sieczkarski and Whittaker 2003).

A summary of current knowledge regarding the major pathways of endocytosis and cellular uptake is shown in figure 1.9.2. Key pathways controlled by Rab proteins are indicated. It should be noted that just the main compartments and intracellular routes are drawn, and other potential routes of entry are by no means precluded.

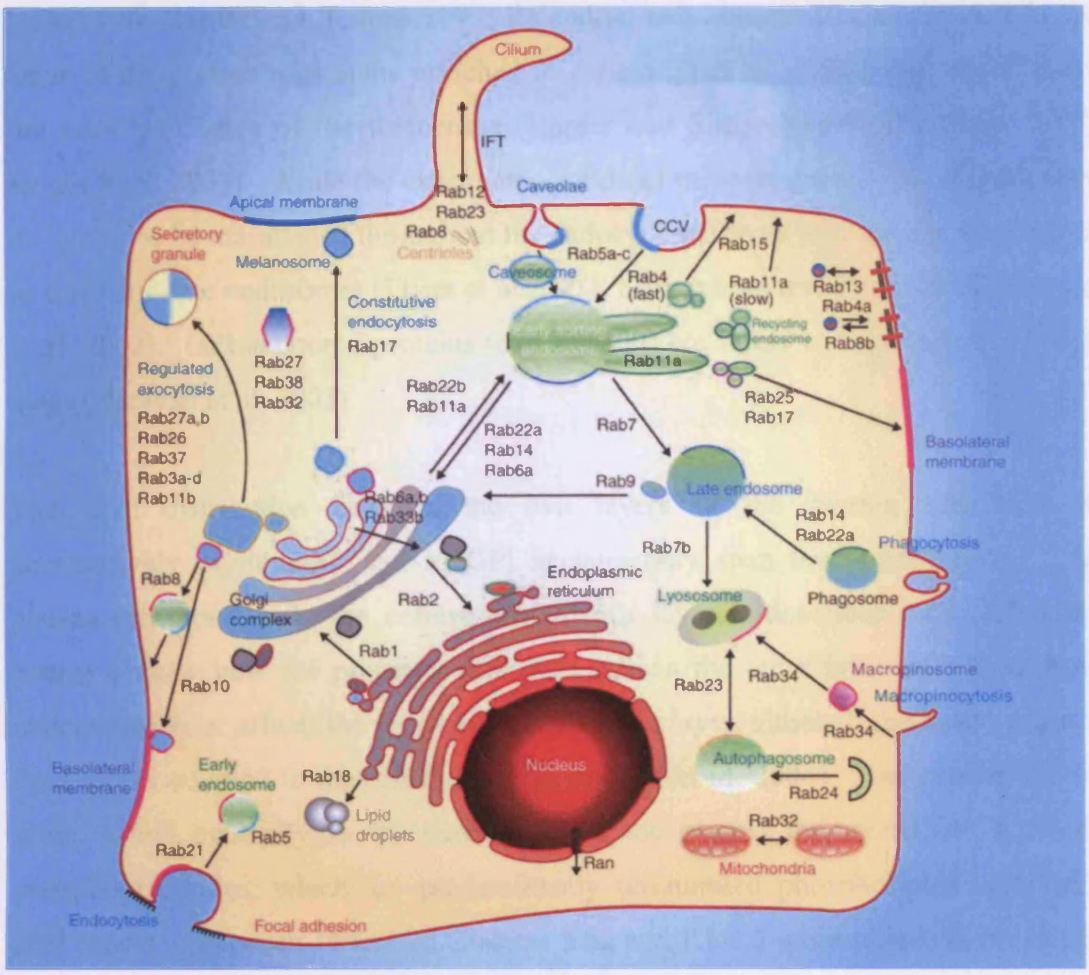


Fig. 1.9.2. The many major points of entry into the cell, each illustrated with some of the known controlling factors. The identity of intracellular compartments can be somewhat fluid and less precisely defined than is apparent from this diagram. However, key controllers such as the Rab proteins are usually found in characteristic locations as shown. From (Schwartz et al. 2007). Abbreviations: CCV, clathrin-coated vesicle; GAP, GTPase activating protein; GDF, GDI dissociating factor; GDI, GDP dissociation inhibitor; GGT, geranylgeranyltransferase; IFT, intraflagellar transport; M6P, mannose 6-phosphate; M6PR, cation-dependent mannose 6-phosphate receptor; MPC, motor protein complex; REP, Rab escort protein; RILP, Ras-interacting lysosomal protein; SNARE, soluble NSF attachment receptor; TIP47, tail-interacting protein 47kD.

1.9.3 Lipid rafts

Other aspects of cellular structure and organisation can strongly influence the entry of viruses into host cells. Many receptors and co-receptors reside in regions of the plasma membrane known as lipid rafts (Simons and van Meer 1988; Simons and Ikonen 1997). As mentioned earlier, these are often found to be sites of caveolar endocytosis, although localisation of a receptor in these domains does not necessarily signify usage of a particular internalisation route (Deckert et al. 1996; Rodgers and

Rose 1996; Harder and Simons 1997; Rajendran and Simons 2005). Lipid rafts are areas of the plasma membrane enriched in certain types of glycolipids, which alters the local properties of the membrane (Harder and Simons 1997; van Meer 2002; Briggs et al. 2003). While the exact composition of these microdomains is fluid, they do appear to be maintained throughout the endocytic pathway, and have been detected in early and late endosomes (Fivaz et al. 2002; Miaczynska and Zerial 2002; Sharma et al. 2003). GPI-anchored proteins (e.g. Tva800) are found to be enriched in these areas (Narayan et al. 2003).

The lipid distribution between the two layers of the plasma membrane is constitutively asymmetric, but as GPI anchors only span the outer leaflet of the plasma membrane only the composition of this layer is described here. It is not certain exactly how the presence of a lipid raft on the outer layer of a membrane interacts with, or affects the properties of, the inner layer, although the inner side of a lipid raft is coupled to the raft by cholesterol (Gri et al. 2004). The principal lipid group found generally in the outer layer of the plasma membrane are types of phosphoglycerides, which are predominantly unsaturated phospholipids with fatty acid chains of between 16 and 22 C-atoms long and 1.1-1.5 unsaturated double bonds per molecule (Harder and Simons 1997). Lipid raft microdomains, however, are enriched for glycosphingolipids and sphingomyelin, which are usually 18-26 C atoms long, with 0.1-0.35 unsaturated double bonds per molecule, and have a higher phase transition temperature (Boggs 1987; Harder and Simons 1997). In this case the phase change is between gel and liquid states, or in other words between an ordered rigid state with similar density and composition to a liquid but structurally related to a solid, and the liquid state, in which components are much more diffuse and disordered. So these areas tend to have the properties of a gel, whereas the rest of the plasma membrane is more fluid (Boggs 1987; Brown and London 1998).

Phosphoglycerides have a small, polar headgroup of phosphate or alcohol, whereas glycosphingolipids have a sugar residue at the 1-OH position. This much larger headgroup in the latter gives increased possibilities for bonding between glycosphingolipids in the same raft (Bittman et al. 1994). The increased length and almost totally saturated nature of the fatty acid chain means that chains can be more tightly packed together, with again more opportunities for van der Waals bonds

between chains, and larger surface areas of chain to interact with cholesterol which can intercalate the phospholipids (Simons and Vaz 2004). GPI anchors would fit well into these tightly packed regions, as the anchor is composed of alkyl fatty acids or ceramide (Ferguson 1999). These anchors do not normally penetrate further than the outer leaflet of the plasma membrane; some ceramide chains may reach into the inner layer, but not beyond that into the cytoplasm. Tighter packing, increased bonding both at the level of the headgroups and between fatty acid chains, and increased ordering resulting from close association of cholesterol result in lipid rafts being regions of rigidity relative to the surrounding areas of the plasma membrane. Lateral diffusion within these areas is slow (Niemela et al. 2007).

Examples of receptors localised here include CCR5 (Yi et al. 2006) and Tva800 (Narayan et al. 2003; Lim et al. 2004). Clustering of receptors in these domains may increase the chances of multiple binding events, localise receptor and co-receptor to the same region of the plasma membrane, and affect both the method and kinetics of entry into the cell. Physical separation of regulatory and effector proteins by partition in raft and non-raft domains can also provide a means of regulation, for example the protein tyrosine kinase $p56^{lck}$ (raft) and its regulator, CD45 (non-raft) (Rodgers and Rose 1996). Within the raft the $p56^{lck}$ is protected from the activity of CD45, which would otherwise de-phosphorylate and activate it (Rodgers and Rose 1996).

1.10 Aims of This Thesis

Over the last few years there have been several breakthroughs achieved in studies of the early stages of retroviral replication, and also an important shift in perception of these early stages. The idea that cells simply need to express the right receptors in order be permissive to infection by a retrovirus is long gone, as is also the idea that cells are largely defenceless against these challenges. The potency of some of the variants of Trim5 against HIV-1 has refocused attention on innate immunity, and inspired ongoing searches for other novel restriction factors.

Shortly before starting these studies, the idea was mooted that the route that a virus takes into the cell could have a profound effect on ability to complete later stages of infection (Schmitz et al. 2004). The question then arose as to whether this could be

true for restriction mediated by Fv1 and Trim5α. Many previous studies had been carried out using VSV-G, so any route of entry dependence would not have been revealed. A new system which could aid characterisation of the early stages of retroviral replication had also just been described (Narayan et al. 2003; Narayan and Young 2004), which took advantage of the fact that ASLV Env can mediate entry through one of two receptors, which subsequently direct the virus down different pathways. It was proposed to use this system first to try and assess route of entry implications for restriction mediated by Fv1 and Trim5α (see chapter 3), and then to study Lv2 restriction, as described in chapter 5 and 6.

During cloning to establish cell lines expressing Tva800 and Tva950, a novel titration curve sparked interest in the relationship of the number of receptors bound by a virus to the ability of that virus to enter the cell, and these studies are reported in chapter 4.

Chapter 2

Materials and Methods

2.1 Cells

2.1.1 Cell Culture

Mus dunni tail fibroblast (Lander and Chattopadhyay 1984), HT1080 (Rasheed et al. 1974), and 293T (Graham et al. 1977) cells were cultivated in 80cm^2 flasks (Nunc) at 37°C under 5% CO_2 in an incubator (LEEC), in complete medium comprising Dulbecco's modified Eagle's Medium (DMEM) (Sigma) supplemented with 10% fetal calf serum (FCS) (PerBio), 100U/ml penicillin-streptomycin (Sigma) and passaged 1:5 twice weekly. The HOS/CD4/CXCR4 cell line (McAllister et al. 1971; Cecilia et al. 1998) was obtained from the AIDS reagent programme (ARP079) and cultivated likewise. HeLa CD4 (Scherer et al. 1953; Maddon et al. 1986), NP2/CD4/CXCR4 (NP2*) (Soda et al. 1999) and U87/CD4/CXCR4 (U87*) (Deng et al. 1996) cells were gifts from Professor Áine McKnight (Queen Mary, University of London), and were cultivated identically except that 5% FCS was used. During passage cells were removed from the plate by incubation at 37°C with Trypsin-Versene (NaCl 8g, KCl 0.2g, Na_2HPO_4 1.15g, KH_2PO_4, 0.2g, EDTA 0.1g, Trypsin 1.25g, phenol red 0.01g to 1L in H_2O pH7.8). Where necessary, medium was supplemented with 1mg/ml G418 (Melford) or 4μg/ml puromycin (Sigma).

2.1.2 Preparation of Tva800 and Tva950-expressing cell lines

Mus dunni cells were seeded at 2.5x10^5 in a 6-well dish (Corning). After 24 hours cells were transduced with pLGateway800IRESG418 or pLGateway950IRESG418 (MLV-based vectors, to introduce genes for production of Tva800 and Tva950. Production of bicistronic mRNA from these plasmids is driven from an MLV LTR. A neomycin gene downstream of the gene for the receptor, and separated by an internal ribosome entry site (IRES), generates resistance to G418 (sections 2.3.1 and 2.6.8). Cells were transduced with these vectors at MOI 0.01 to 10. After 72 hours cells were passaged and replated at a density of 4x10^5 per well. Medium was then supplemented

with G418 at 1mg/ml and replaced daily. After >10 days of selection and considerable cell death, colonies were isolated by sealing a cloning ring (Sigma) around a cell clump using silicone grease. Cells within the ring were removed using 100 µl trypsin and replated in single wells of a 24-well dish (Nunc). Cells were grown for a further 2 weeks before transfer to 80cm^2 flasks and further passage as above.

2.2 Analyses

2.2.1 Cell sorting

Mus dunni or HeLa CD4 cells were seeded at 5x10^4 in a 12 well dish and 24-hours later were transduced with pLGateway**X**IRESYFP, in which a bicistronic mRNA encodes a gene of interest (**X**) and downstream of an IRES, yellow fluorescence protein, YFP. After 72 hours cells were harvested by incubation at 37°C with trypsin-versene and resuspended in complete DMEM at a density of less than 2x10^7 cells per ml. Cells were selected for YFP expression using a Dako Cytomation MoFlow (Dako Ltd), replated as a pool of clones in 25cm^2 tissue culture flasks (Nunc), or an average of 1-cell per well, to be grown as single cell clones in a 96-well plate (Nunc), and incubated at 37°C, 5% CO_2 until confluent. From then on cells were transferred to larger culture areas until 80cm^2 flasks (Nunc) were reached, after which passage was as described above. Sorted in this way were NP2** cells expressing Trims 1, 18 and 34.

2.2.2 Fluorescence activated cell sorting (FACS)

Cells were harvested by incubation with Trypsin-Versene at 37°C, resuspended by repeated pipetting and fixed in 3mL 7% formaldehyde in PBS. Samples were centrifuged at 1200xg for 8 minutes at 4°C, and resuspended in 50µl PBS. Analysis of 1-colour assays was by FACSCalibur (Becton Dickinson) and 2-colour assays by LSR II (Becton Dickinson). Cells are taken up by the FACS machine in a thin stream, and analysed in single file. A laser beam is directed at the stream, and the scattering of the light caused by cells is detected by a photocell. Scattering data were analysed using FlowJo software v.8.4.5 (Tree Star Inc.). Cell populations were grouped

according to YFP or GFP fluorescence and ratios of the fluorescent populations analysed as described (Bock et al. 2000) and as follows.

If some cells in a sample express just one fluorescent protein, then two populations will be detected; those that express the protein and those that do not. If cells can potentially express two colours (e.g. YFP and GFP), the sample will divide into 4 populations; those that express just YFP, just GFP, both and neither. The cells that have been transduced with the gene of interest are YFP positive. Those that have been transduced with the challenge virus are GFP positive. The population of cells that are YFP positive lie in the upper right and lower right hand quadrants. The cells that are GFP positive lie in the upper two quadrants of the FACS profile. For a sample of cells, the proportions that lie in each of the 4 quadrants can be compared and analysed to give information about potential interactions between the gene of interest and the challenge virus.

For example, in fig. 2.2, YFP fluorescence is shown on the x, or FL1-H axis, and GFP fluorescence is shown on the y, or FL2-H axis. The proportion of cells in each quadrant is given as a percentage and shown in the corner of the quadrant. The overall proportion of cells successfully transduced with the gene of interest (i.e. FL1-positive) is 3.39% (top right) + 46.6% (bottom right), or 49.99%. The overall proportion of cells successfully infected by the challenge virus (i.e. FL2-positive) is 13% (top left) + 3.39% (top right) = 16.39%.

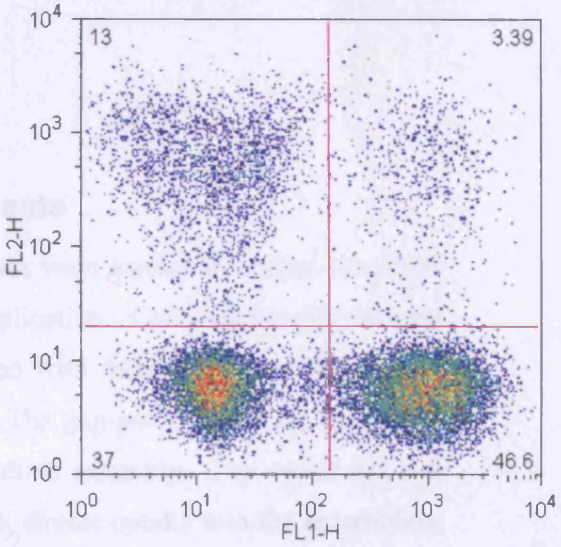


Fig. 2.2 A typical 2-colour FACS profile

Comparing the percentage of cells infected with the challenge virus that carry the gene of interest and the percentage of cells that do not, gives a ratio of between 0 and 1. The percentage of cells infected with the challenge virus that do not carry the gene of

interest is 13/(13+37), or 26%. The percentage of cells infected with the challenge virus that were transduced with the gene of interest is 3.39/(3.39+46.6), or 6.78%. If these two ratios are compared by calculating the fold-difference between them, a figure can be obtained between 0 and 1 that is indicative of whether the exogenous gene of interest is having an effect on whether the virus can replicate or not. The ratio obtained for the restriction of N-MLV by rhesus macaque Trim5α is 0.3, and from titration curves this is considered to be restriction This sets an upper limit on the value of the ratio that can be considered a restriction, as stronger restrictions (e.g. human Trim5α against N-MLV) generate a ratio of less than this. A value between 0.7 and 1 is considered to show no restriction, as this is the value obtained with factors that did not restrict when meansured by titration curves. Ratios between 0.3 and 0.7 are an intermediate phenotype that needs to be interpreted with caution (Yap *et al.* 2005; Ohkura *et al.* 2006; Yap 2008). For the figure above, the value would be 0.0678/0.26, or 0.3. This indicates that the gene of interest is restricting viral replication in the YFP positive cells for the sample shown.

2.2.3 Microscopy

Slides were prepared as described in section 2.4.6, and were viewed on a Deltavision Olympus IX70 inverted microscope with 100x lens and initially analysed using Softworx image acquisition software (Applied Precision). Where further study was necessary, images were viewed and manipulated using Adobe Photoshop CS2 (Adobe Systems).

2.3 Viruses

2.3.1 Env, Gag-pol and vector components

All experiments described here that involve viruses were carried out using retroviral vectors, which only undergo a single round of replication. Cells that readily take up plasmids (in this case 293T cells) are transfected with three separate components: gag-pol, env, and vector (Soneoka et al. 1995). The gag-pol and env are expressed from CMV promoters in the transfected cell and drive assembly. The vector has a ψ signal between the LTR and inserted gene, which directs uptake into the assembling

progeny virions. The plasmids do not have a packaging signal, and so no carry over of these components occurs.

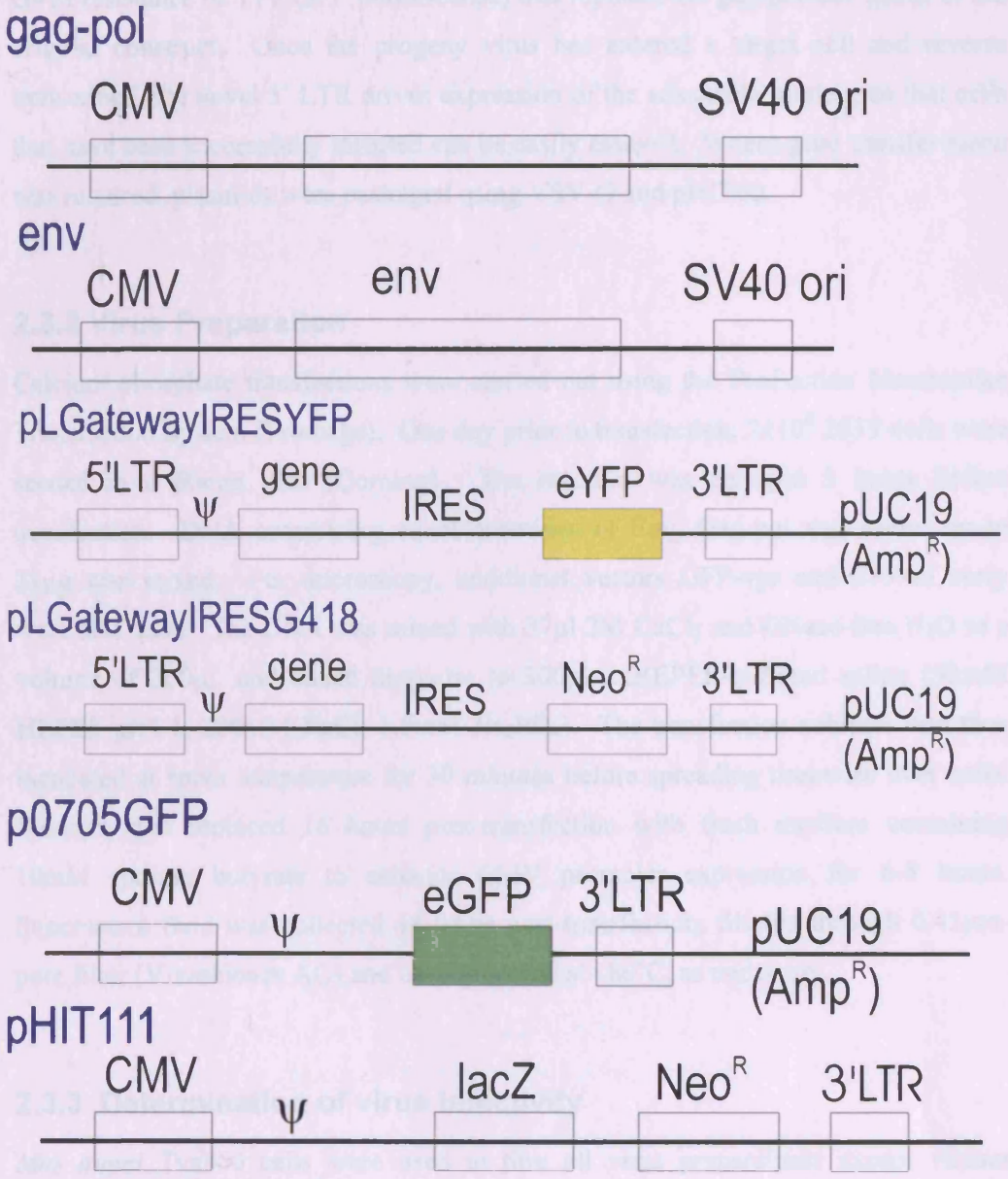


Fig 2.3.1 Schematic representations of vectors used in the production of virions. Gag-pol constructs used were p8.91 (HIV-1) (Naldini et al. 1996) and pHIT60 (Moloney MLV) (Soneoka et al. 1995). Env constructs include VSV-G (Bock et al. 2000), pHIT456 (amphotropic envelope) (Soneoka et al. 1995), MCR (molecular clone restricted), MCN (molecular clone non-restricted) (Schmitz et al. 2004) and ASLV-A Env (pCB6), which was a gift from P Bates. Packaged vectors into YFP-gateway were $Fv1^n$, $Fv1^b$ (Lindemann et al. 1997), Trim5α (Yap et al. 2004), Tva800, Tva950, HA-Tva800, HA-Tva950 and Rabs 5, 7, 9, 11 and the corresponding dominant negative constructs of these proteins. G418 gateway was based on pLXSN to generate two constructs, encoding Tva800 and Tva950. Reporter vectors used were p0705GFP, a gift from D Lindemann, and pHIT111 (Soneoka et al. 1995) (both MLV-based), and CSGW (Bainbridge et al. 2001) (HIV-based). Viral markers were GFP-vpr (McDonald et al. 2002) and S15-mC (Campbell *et al.* 2007b).

The vector has a packaging signal, retroviral LTR and a selectable marker (commonly G418 resistance or YFP/GFP fluorescence) that replaces the gag-pol-env genes of the original construct. Once the progeny virus has entered a target cell and reverse transcribed, the novel 5' LTR drives expression of the selectable marker, so that cells that have been successfully infected can be easily assayed. Where gene transfer alone was required, plasmids were packaged using VSV-G and pHIT60.

2.3.2 Virus Preparation

Calcium phosphate transfections were carried out using the ProFection Mammalian Transfection System (Promega). One day prior to transfection, $2x10^6$ 293T cells were seeded in a 60mm dish (Corning). The medium was changed 3 hours before transfection. DNA comprising equal quantities of Env, Gag-pol and vector up to 21μg was mixed. For microscopy, additional vectors GFP-vpr and S15-mCherry were also used. The DNA was mixed with 37μl 2M $CaCl_2$ and DNase-free H_2O to a volume of 300μl, and added dropwise to 300μl 2xHEPES-buffered saline (50mM HEPES pH7.1, 280mM NaCl, 1.5mM Na_2PO_4). The transfection solution was then incubated at room temperature for 30 minutes before spreading dropwise over cells. Medium was replaced 16 hours post-transfection with fresh medium containing 10mM sodium butyrate to enhance CMV promoter expression for 6-8 hours. Supernatant fluid was collected 48 hours post-transfection, filtered through 0.45μm-pore filter (Vivascience AG) and used or stored at –80°C, as necessary.

2.3.3 Determination of virus infectivity

Mus dunni Tva800 cells were used to titre all virus preparations except viruses pseudotyped with MCR, in which case NP2* cells were used. $4x10^4$ cells were seeded per well. 24 hours later cells were transduced with increasing volumes of virus carrying eGFP or eYFP vector. Analysis was 3 days later by FACS, to determine the percentage of GFP or YFP positive cells. Titre was calculated using a formula derived for MLV by Dr. M Bock based on the Poisson distribution. The MOI is proportional to $2\ln(1-x)$ where x represents the proportion of cells GFP or YFP

positive ($0 \leq x \leq 1$). The number of infectious particles per ml can then be calculated by multiplying MOI by number of cells initially infected, and the dilution factor.

2.4 Assays

The assays detailed below were performed in 12-well dishes (Nunc) unless stated otherwise, and analysis was by flow cytometry to assess levels of eGFP or eYFP fluorescence, except for cells prepared for microscopy.

2.4.1 Infectivity assay

$5x10^4$ cells were seeded in 12-well dishes, and allowed to settle. 24 hours later cells were incubated with DMEM + chemical reagents as shown in table 2.4.1 below, for between 30 mins - 1 hour. Between 100-1000µl virus was then added in DMEM with the reagent, adjusted to keep the reagent at the appropriate concentration.

Name	Provenance	Diluent	Stock	Usage
Amantadine	Sigma	ddH_20	150mM	1mM
Bafilomycin A1	Roche	ddH_2O	5µM	50nM
Chloroquine	Sigma	ddH_2O	1mM	2.5µM
Chlorpromazine HCl	Sigma	ddH_2O	10mg/ml	10µg/ml
Genistein	Acros Organics	DMSO	25mg/ml	5µg/ml
NH_4Cl	Sigma	ddH_2O	500mM	40mM

Table 2.4.1 Chemicals used to affect cellular processes, which were assayed for their effects on viral infectivity. Reagents were added at concentrations shown in the 'usage' column in DMEM.

2.4.2 Abrogation assay

Viruses were created via the calcium phosphate method described above with either LacZ or eGFP vector. Cells were seeded at $5x10^4$ per well. After 24 hours medium was removed and cells incubated with either supernatant from 293T cells that had either not been transfected (mock) or viruses delivering LacZ vector at an MOI of 10. 3 hours later this was removed and cells were incubated with eGFP virus at MOI 1 in 1mL DMEM. Cells were analysed 3 days later.

2.4.3 SiRNA

HeLa CD4 cells were plated at $5x10^4$ cells per well, and transduced 24 hours later with pLSiT1SN, a vector encoding anti-Trim 1 SiRNA, at MOI greater than 5. After 48 hours cells were passaged 1:10 into a 12 well plate, and after a further 24 hours were challenged with MCR-pseudotyped viral eGFP vector. Analysis was carried out after a further 3 days.

2.4.4 NH_4Cl treatment of cells

Mus dunni cells were seeded at $5x10^4$ per well. After 24 hours the medium was removed and replaced by complete DMEM + 40mM NH_4Cl. After 1 hour to equilibrate, the medium was removed, and replaced by ASLVenv pseudotyped NB-MLV (MOI 2-3) in DMEM + 40mM NH_4Cl. Virus was allowed to bind for 1 hour by placing the plates at 4°C on an orbital mixer (Denley) after which the cells were washed once with PBS + 40mM NH_4Cl, and then incubated at 37°C in complete DMEM + 40mM NH_4Cl. At preset time-points between 1-8 hours, this medium was replaced by complete DMEM. Analysis was 3 days later.

2.4.5 Inhibition of infection by SUA-rIgG or empty vector

Cells were prepared that expressed Tva800 or Tva950 as described in 2.1.2, or as follows. *Mus dunni* cells were plated at a density of $5x10^4$ in a 12-well dish, and 24 hours later were transduced with pLgatewayTva800IRESYFP or pLgatewayTva950IRESYFP at MOI between 0.1 to 20. The SUA-rIgG is a fusion protein made of ASLV-A SU fused in-frame to a rabbit immunoglobulin constant region (Zingler and Young 1996). Supernatant containing SUA-rIgG was harvested from 293T cells transfected with SUA-rIgG vector, a gift of J Bruce. SUA-rIgG will bind to Tva800 and Tva950, but is not able to initiate fusion. In order to assess the effect of inhibition on infection, samples containing preset constant levels of eGFP vector mixed with increasing volumes of SUA-rIgG or empty vector were prepared as shown in table 2.4.5. The eGFP vector was ASLV Env pseudotyped NB-MLV with p0705GFP, and the empty vector the same, but without p0705GFP.

Well	eGFP vector (µl)	SUA-rIgG or empty vector (µl)	PBS (µl)
neg	0	0	510
1	10	0	500
2	10	5	495
3	10	10	490
4	10	20	480
5	10	50	450
6	10	100	400
7	10	200	300
8	10	500	0

Table 2.4.5 Inhibition of infection by SUA-rIgG. A preset volume of eGFP vector was mixed with increasing levels of SUA-rIgG in competition for binding to Tva800. Reagents were mixed on ice before being added to cells.

These preparations were added to cells, and left for 6 hours, after which the medium was replaced with fresh complete DMEM. Analysis of infection by eGFP fluorescence was after 3 days.

2.4.6 Immunofluorescence

Glass coverslips (Scientific Lab Supplies) were sterilised by autoclave, and placed at the bottom of wells in a 12-well dish (Nunc). HeLa CD4, U87*, NP2*, d800 or d950 cells were plated at between $1x10^5$ and $5x10^5$ cells per well, and allowed to attach overnight. 24 hours later cells were cooled to 4°C, then incubated with virus at MOI between 1-5. After binding at 15°C for 2 hours while centrifuging at 1200xg (experiments with HeLa CD4, U87* and NP2* cells) or binding at 4°C for 1 hour with shaking (experiments with d800 and d950 cells), 1mL pre-warmed DMEM ± 40mM NH_4Cl was added to each well, and infection allowed to proceed for preset time periods. All virions used were HIV-1 based, and additionally carried GFP-vpr, which binds to the viral RNA in high copy number, making the virions visible as puncta of GFP fluorescence. HeLa CD4, U87* and NP2* cells were infected with VSV-G and MCR pseudotypes which were produced in cells that also expressed S15-mC, which stains the virions with mCherry (red) as they exit the producer cell. After binding and

fusion with the membrane of the target cell, this red signal is lost, allowing an assessment of fusion. The d800 and d950 cells were infected with ASLV-A Env pseudotypes of HIV-1. In order to stop the infection, cells were washed 1x with PBS and incubated with 4% paraformaldehyde in PBS at room temperature for 15 minutes. Cells were then permeabilised in 0.2% Triton X-100 (BIORAD) in PBS for 15 minutes, blocked for 20 minutes with 1% BSA in PBS, stained with primary antibody and secondary as appropriate (see table 2.4.6), both diluted in 1% BSA in PBS. When possible, cells were also stained with DAPI (PerBio Ltd) used at 10μg/ml in PBS. Finally, coverslips were prised off the plate and placed cell-side down in 20μl Citifluor (Agar Scientific Ltd) on 76mm x 26mm x 1.0mm slides (Fisher Scientific) and sealed around the edge with nail varnish.

Where the primary detection agent was SUA-rIgG, the following procedure was undertaken. The dish with cells on was cooled to 4°C and maintained on ice until fixed. After cooling, cells were incubated for 1hr at 4°C with 200μl SUA-rIgG-containing supernatant prepared as follows: a 6mm dish of 293T cells plated for transfection as described in 2.3.2 was transfected with 10μg SUA-rIgG DNA (Zingler and Young 1996). 24 hours later cells were incubated for 6 hours with 10mM sodium butyrate, after 48 hours the supernatant was harvested and filtered through a 20μM-pore filter (Vivascience AG), and used immediately or frozen at -80°C as required.

Antibodies used:

	Target	**λ**	**Source Animal/Type**	**Dilution**	**Company**
1°	EEA1		Goat polyclonal	1/200	Santa Cruz
1°	Lamp3		Mouse monoclonal	1/200	Santa Cruz
1°	SUA-rIgG		SU of ASLV Env fused to rabbit heavy chain IgG	100μl of supernatant	
2°	Alexa Fluor	594	Goat anti-rabbit	1/800	Invitrogen
2°	Alexa Fluor	594	Rabbit anti-mouse	1/800	Invitrogen
2°	Alexa Fluor	594	Donkey anti-goat	1/800	Invitrogen

Table 2.4.6 Primary and secondary antibodies used for immunofluorescence.

2.5 Protein Analysis

2.5.1 Polyacrylamide gels

Samples were prepared as follows: 60mm dishes of the cells to be assayed were seeded and grown to confluency. 1µl benzonase (Roche) in 500µl 95°C 1xSDS loading buffer (50mM Tris-HCl, 100mM DTT, 2% SDS, 0.1% Bromophenol Blue, 10% glycerol) was added, and cells were removed from the plate using a cell scraper (Corning). Samples were incubated at 100°C for 10 minutes, then centrifuged at >12,000xg for 10 minutes. The supernatant was removed, and 20µl applied to the gel. 5µl PageRuler Prestained protein ladder (Fermentas) was run alongside samples, with markers at 170, 130, 100, 70, 55, 45, 35, 25, 15, and 10kDa.

Analytical denaturing gels were run on the mini-PROTEAN II system (BIO-RAD). Gels consisted of 1.5-2cm stacking gel of 5% acrylamide (stock 30% Acrylamide/Bis 37.5:1, BIO-RAD), 124mM Tris-HCl pH 6,8, 0.1% ammonium persulphate, 0.1% SDS and 10µl N, N, N', N'-Tetra-methyl-ethylenediamine (TEMED, Sigma). The separating gel was composed of 10% acrylamide stock, 375mM Tris, pH8.8, 0.1% SDS, 0.1% ammonium persulfate and 4µl TEMED. Gels were run at 100V for up to 2 hours in running buffer (0.1% SDS, 25mM Tris, 250mM glycine, pH 8.3).

After separation, proteins were transferred to an Immobilon-P membrane (Millipore) using a semidry electrotransfer apparatus (Ancos). The membrane was presoaked in methanol, then in transfer buffer (20% methanol, 2.9g glycine, 0.36mL SDS, 5.8g Tris in 1l) surrounded by 4-6 pieces of blotting paper and run for 1 hour at 20V. The membrane was blocked in 5% non-fat milk in 0.1% polyoxyethylene-sorbitan monolaurate (Tween 20)/PBS for a minimum of 2 hours, or overnight.

2.5.2 Antibodies used for Western blot

Primary and secondary antibodies used to probe Western blots were as follows:

	Target	Source Animal/Type	Dilution	Company
1°	CD4	Sheep polyclonal sera (404)	1/500	ARP
1°	CXCR4	Rabbit polyclonal	1/1000	Merck
1°	HA	Rabbit polyclonal	1/5000	Sigma
1°	α-Tubulin	Mouse monoclonal	1/5000	Sigma
1°	GAPDH	Mouse monoclonal	1/4000	Chemicon
1°	GFP (also YFP)	Mouse monoclonal	1/10000	Santa Cruz
2°	Anti-Rabbit HRP	Goat polyclonal	1/20000	Pierce
2°	Anti-Mouse HRP	Rabbit polyclonal	1/10000	Pierce
2°	Anti-Sheep HRP	Donkey polyclonal	1/10000	Sigma

Table 2.5.2 Primary and secondary antibodies used for Western blotting.

The primary antibodies shown in table 2.5.2 were diluted in 5mL 5% non-fat milk in 0.1% Tween-20/PBS and bound to the membrane for 1-2 hrs at room temperature on a suspension mixer (Luckham Ltd) except for antibodies to CD4 and CXCR4, which were bound overnight at 4°C. Membranes were washed 3x in 0.5% non-fat milk in 0.1% Tween-20/PBS for 5 mins, then secondary antibody was bound, diluted and incubated as for the primary to the concentrations shown in table 2.5.2. After 2 washes in 0.5% non-fat milk in 0.1% Tween-20/PBS and 1 wash in PBS, the level of HRP antibody bound to membranes was revealed by incubation in 2mL ECL reagent (Chemilucent ECL, Millipore) and then exposure on KODAK Biomax film for between 5 seconds and 5 minutes, as appropriate.

For blots that were reprobed, stripping of the initial primary and secondary antibodies was done by incubation in Restore Western Blot stripping buffer (Pierce) at 37°C for 15 minutes. Removal of antibody was assessed by incubation with ECL and exposure to film, as before. Membranes were blocked again in 5% non-fat milk in 0.1% Tween-20/PBS overnight, and re-probed.

2.6 DNA Purification and Manipulation

2.6.1 Agarose DNA gels

DNA samples were separated by size using horizontal slab agarose gels. 1% (w/v) agarose (Melford) was dissolved in TBE (0.1M Tris, 0.1M Boric Acid, 5mM EDTA in ddH_2O) and 4µl Ethidium Bromide (BIO-RAD). Samples were applied in 10% loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in H_2O) and run at 80V. DNA size markers used were either HaeIII digest of φX174, HindIII digestion of λ or Smartladder (Eurogentec).

2.6.2 DNA purification from agarose gels

In order to purify PCR products for further cloning, fragments were separated and identified by size on agarose gels, then cut out using a scalpel and the DNA purified using QIAquick gel extraction kit (Qiagen), according to the manufacturer's protocol. Briefly, the sample is dissolved in a high-salt buffer and bound to a silica membrane. The sample is washed, then eluted in low-salt buffer.

2.6.3 Quantification of DNA

The concentration of DNA samples was determined using a spectrophotometer (Biophotometer, Eppendorf). Samples are automatically quantified based on the premise that an absorbance of 1.0 at 260nm in a 1cm cell is from 50µg/ml of double-stranded DNA. The purity of samples is estimated from the ratio of the absorbances 260 and 280nm; an A_{260}:A_{280} of >1.75 is assumed to be free from protein contamination.

2.6.4 PCR

Taq (Abgene Ltd) and PfuUltra (Stratagene) amplifications were carried out according to the manufacturers instructions using 0.2mL tubes (Alpha Laboratories) using a PTC 100 thermal cycler (MJ Research).

Programmes used were as follows:

For amplification of DNA fragments, samples were heated to 95°C for 2 minutes, then subjected to 25 cycles of 95°C 1 minute, 60°C 30 seconds, 72°C for 1 minute per kb of fragment amplified, 10 minutes at 72°C and cooled to 4°C.

For QuikChange mutagenesis 18 cycles were used as above, except with extension times of 1 minute per kb of plasmid, plus an additional 2 minutes.

For insertional mutagenesis samples were heated to 94°C for 2 minutes, then 30 cycles of 94°C for 1 minute, 60°C for 2 minutes, 72°C for 1 minute 30 seconds, then 72°C for 10 minutes and cooled to 4°C.

2.6.5 Quantitative PCR

$2x10^5$ cells were plated out in 60mm dishes (Corning). After 24 hours cells were infected at MOI of between 2 to 5 at 4°C for 1 hour with shaking on a rotary mixer (Denley). Prewarmed medium was added at 37°C and incubated for either 6 or 18 hours incubation at 37°C. The total DNA of the cells was extracted after 6 or 18 hours using QIAamp DNA mini kit (QIAGEN). HIV-1 early reverse transcription products were detected using primers to amplify the RU5 region in DNA extracted at 6 hours. These primers were designated HIV eF and HIV eR. For DNA extracted at 18 hours, late products of reverse transcription were quantified using primers HIV F 2LTR and HIV R 2LTR (Yap et al. 2006). Samples were normalised for cell number by using an actin positive control (o-A1 and o-A3). All primer sequences are given in section 2.6.15. Analysis was carried out on 10µl (150ng) of the extracted DNA with 70nM primer and 1x SYBR Green mix (Abgene) in a volume of 25µl, using an ABI Prism 7000 Sequence Detection System (Applied Biosystems) and the software as provided by the manufacturer. The program consisted of an initial incubation at 50°C for 2 min, then 95°C for 15 min, 40 cycles of 95°C for 15 seconds and 60°C for 1 min.

2.6.6 PCR cloning

Primers were designed to amplify genes of interest using published sequences (table 2.6.6). A CACC tag was added to the 5' end of the forward primer to permit cloning into TOPO D-entry vector (Invitrogen). Template DNA was prepared from harvested cells using DNeasy kit (Qiagen).

Common Name	GeneID
CD4	920
CXCR4	7852
Rab5	5868
Rab7	7879
Rab9	9367
Rab11	8766
MSV	2193426
$p56^{lck}$	3932

	GenBank ID
Tva800	403161
Tva950	403162

Table 2.6.6 Published sequences used to design primers and to check sequences of cloned products.

2.6.7 QuikChange PCR

Mutation of 1-4 nucleotides was carried out using the QuikChange mutagenesis kit (Stratagene), according to the manufacturer's protocol. Briefly oligonucleotides between 24-30 bases in length were designed that flanked the nucleotide(s) to be changed, incorporating the desired mutation. PCR amplification would then be carried out using the programme described above, with a reaction typically consisting of 40ng template DNA, 125ng of each primer, 200μM dNTPs, 1μl (2.5U) PfuUltra polymerase and 5μl 10x polymerase buffer made up to 50μl with ddH_2O. After amplification samples were cooled, then digested and incubated at 37°C for 1 hour with DpnI, so removing the methylated template DNA. Samples were then concentrated by sodium acetate/ethanol precipitation and resuspended in 5μl TE (10mM Tris, 1mM EDTA pH8.0). An aliquot of 2μl was used to transform 50μl One Shot TOP10 cells (Invitrogen). Successful transformations were selected overnight on kanamycin (Sigma) or nafcilin/ampicilin (Sigma) plates, colonies picked, and the mutation confirmed by DNA purification and sequencing.

2.6.8 Cloning via the Gateway system

The Gateway cloning system (Invitrogen) is based on phage λ, and is designed to allow genes of interest to be easily transferred between different vector backbones. First a PCR product is subcloned into pENTR-D-TOPO (entry vector) in a directional reaction. Two sites, attL and attR, flank a lethal *ccdB* gene in this vector, which is replaced by the PCR product. A second type of vector, the destination vector, carries a selective gene marker for either YFP fluorescence (pLgatewayIRESYFP) or G418 resistance (pLgatewayIRESG418). A recombination reaction catalysed by LR clonase enzyme replaces *ccdB* in the destination vector with the gene of interest (PCR product) from the entry vector. Transformation of the products of this reaction into TOP10 chemically competent E.coli cells and selection by antibiotic marker selects against cells carrying the original destination vector with the lethal *ccdB* gene, and for successful recombinants carrying the gene of interest. Vectors prepared by this method include all cloned into pLgatewayIRESYFP (e.g. Tva800-YFP), pLgatewayTva800IRESG418 and pLgatewayTva950IRESG418.

2.6.9 DNA precipitation

DNA samples were concentrated by precipitation with 2µl 3M sodium acetate pH5.2 and 2.5 volumes of 100% ethanol, followed by at least 20 minutes incubation at -80°C. DNA was pelleted by centrifugation at 16,000xg for 30 minutes at 4°C and washed once with 70% ethanol.

2.6.10 Transformation

Up to 5µl DNA was incubated with 50µl TOP10 cells (Invitrogen) on ice for 30 minutes before heat shock at 42°C, recovery on ice for 2 minutes then incubation for 1 hr with 250µl SOC medium (20g bacto-tryptone, 5g bacto-yeast extract, 8.55mM NaCl, 2.5mM KCl, 10mM MgCl2, 20mM glucose made up to 1litre) with shaking. Cells were then spread on LB agar plates made up with the appropriate antibiotic (50µg/ml kanamycin or nafcilin/ampicilin (Sigma) and incubated overnight at 37°C.

2.6.11 Selection of colonies

Bacterial samples were spread on agar plates with 50μg/ml selection agent (kanamycin or nafcilin/ampicilin) and grown overnight. Colonies were selected and grown in 2mL LB broth (10g bacto-tryptone, 5g bacto-yeast extract, 10g NaCl made up to 1 litre, pH7.5) in half-test tubes at 37°C with shaking.

2.6.12 Preparations of DNA

1.5mL of culture from colonies transformed and selected as described above was pelleted and DNA purified using Purelink Quick Plasmid Miniprep kit (Invitrogen) according to the manufacturer's protocol. Briefly, cells are collected through centrifugation, lysed, neutralised, and chromosomal DNA pelleted. Plasmid DNA is bound to a silica column, washed, and eluted.

Large-scale (midi) preparations of DNA were carried out using a similar procedure using the Purelink Quick Midi-prep kit (Invitrogen). 100μl frozen or mini-prep culture was used as a starter for 100mL LB broth grown overnight at 37°C with the appropriate antibiotic, Ampicilin/Nafcilin or Kanamycin at 50μg/ml, with shaking. Cells were harvested and subsequently DNA was purified using the according to the manufacturer's protocol. Briefly, cells were centrifuged, lysed, neutralised and chromosomal DNA pelleted. DNA is bound to an anion exchange column, impurities washed away with buffer at neutral pH, and the DNA eluted with a high-salt buffer. Eluted DNA is then precipitated and washed with ethanol.

2.6.13 DNA sequencing

Sequencing reactions were carried out using Big Dye Terminator Sequencing kit (v3.1) (Applied Biosystems), according to the manufacturer's protocol. Briefly, 350ng sample DNA, 3.2pmol primer, 3μl reaction buffer and 2μl Big Dye were mixed in 0.2mL PCR tubes. Samples were subjected to 96°C for 1 minute prior to 30 cycles of 96°C for 10 seconds, 50°C for 4 seconds 60°C for 4 minutes. DNA was concentrated using sodium acetate/ethanol precipitation before analysis on a MegaBACE capillary sequencer (Pharmacia). Plasmids and samples longer than 2kb were sequenced by GeneService Ltd (Medical Solutions plc).

2.6.14 Preparation of mRNA

~$5x10^6$ cells were harvested and pelleted by spinning at 1,000xg for 10 minutes. mRNA was then extracted and purified using RNeasy mini kit (Qiagen) according to the manufacturer's protocol. Briefly, cells are lysed and homogenised in an RNase-inactivating denaturing buffer, RNA is bound to a membrane, washed, and eluted.

2.6.15 Primers

Primer Name	Sense/ Antisense	Sequence
EG4	S	CACCATGGCTAGTCGAGGCGCAAC
EG5	AS	TTAGTTACTACAACACTGATTCC
EG6	S	CACCATGACCTCTAGGAAGAAAGTG
EG7	AS	AGATCTTCAGCAACTGCAGCTTTCTG
EG11	S	CTGGAGTCGGGAAGAACTCACTCATGAACCAG
EG12	AS	CTGGTTCATGAGTGAGTTCTTCCCGACTCCAG
EG13	S	CTCCATCCCCCCGTCTCTCCCCC
EG14	AS	CCAACGACTGATTAACTCTACGTAC
EG15	S	CACCATGGGCACCCGCGACGACGAG
EG16	AS	TTAGATGTTCTGACAGCACTGC
EG17	S	CACCATGGCAGGAAAATCTTCAC
EG18	AS	TCAACAGCAAGATGAGCTAGGC
EG21	AS	CGAAGCACTAGGCTGTTTTTGCCAACAGC
EG22	S	GCTGTTGGCAAAAACAGCCTAGTGCTTCG
EG23	S	GGAGTTGGGAAGAATTCACTTATGAAC
EG24	AS	GTTCATAAGTGAATTCTTCCCAACTCC
EG25	S	CTGGTGTTGGAAAGAATAATCTCCTGTCTCG
EG26	AS	CGAGACAGGAGATTATTCTTTCCAACACCAG
EG27	S	GGTGGAGAAGCGACCCCTGCGGG
EG28	AS	ATCTTTAGCCCAGTGCCCC
EG39	S	CCGCCCCCTTCACCATGGCGCGGCTGCTGCC

EG40	AS	GGCAGCAGCCGCGCCATGGTGAAGGGGGCGG
EG41	S	TTACCGGACCCGTTACCGGTCAC
EG42	AS	GGTGAAGGGGGCGGCCGCGGAGCCTG
EG43	S	CCCCCTTCACCAGGGAGAGGATGCTG
EG44	AS	CAGCAGCCGCGCCCTGGTGAAGGGGG
EG45	AS	GCGATCCGCTCGACATCTTTCC
EG46	S	GCTGTTGGCAAATCAAGCCTAGTGCTTCG
EG47	AS	CGAAGCACTAGGCTTGATTTGCCAACAGC
EG48	S	TACCCTTACGATGTTCCTGATTACGCTAAC GGGTCCGGTAACGGTTCTTTGTCCCG
EG49	AS	AGCGTAATCAGGAACATCGTAAGGGTAAC CGGTCACGTTACCGGGCAGC
EG55	S	AGGGCGCGGCTGCTGCCC
EG56	AS	TAACACTCCCCGTGGGCAC
EG57	S	ATGGAGGGGATCAGTATATACAC
EG58	AS	TTAGCTGGAGTGAAAACTTGAAG
EG67	AS	GAGGCTTAGGGTGTACAAAGGGCTTG
EG68	S	CGACGGGCGGGACGAGTGGGGCTGCG
EG69	AS	GGCGGTAATACGGTTATCCACAGAATC
EG70	S	CGAATTAATGTGAGTTAGCTCACTC
EG71	AS	GAGTGAGCTAACTCACATTAATTGCG
EG72	S	GATTCTGTGGATAACCGTATTACCGCC
EG73	AS	GAACAGCTCCTCGCCCTTGCTCACCAT
EG74	S	CAACGAGAAGCGCGATCACATGGTC
EG79	S	GTCAAGCCCTTTGTACACCCTAAGC
EG80	AS	GTTTGCAAGCAGCAGATTACGCGCAG
EG81	S	CGTTGGCCGATTCATTAATGCAGC
EG82	AS	GGACGAGCCCCCAATGAAAGACC
EG83	AS	CGGGATAATACCGCGCCACATAGC
EG84	AS	GTTTGGTATGGCTTCATTCAGCTCC
EG85	S	CACCTACGGCAAGCTGACCCTGAAG
EG86	S	CAAGATCCGCCACAACATCGAGGAC
EG90	AS	CGGTGCGGGCCTCTTCGCTATTACGCC

EG91	S	TCCGACTTGTGGTCTCGCTGTTCC
EG92	S	CAACGCTCCGGCTCAGGTGTCAGG
EG93	S	ATGGGCTGTGGCTGCAGCTCACAC
EG94	AS	TCAAGGCTGAGGCTGGTACTGG
EG95	S	CACCATGGGCTGTGGCTGCAGCTC
EG96	S	CCGCCCCCTTCACCGACGCGCGGCTGCTGCCC
EG97	AS	GGGCAGCAGCCGCGCGTCGGTGAAGGGGGCGG
TS221	S	CACCATGGCGCGGCTGCTGCC
TS222	AS	TCAGTCCCATCTCACCAGCTC
TS223	AS	TCAGGAGAACAAGTCTGCCTG
T- CD4	S	CACCATGAACCGGGGAGTCCCTTTTAG
CD4	AS	CGGGATCCTCAAATGGGGCTACATGTCTTC
HIV eF	S	TCTGGCTAACTAGGGAACCCA
HIV eR	AS	CTGACTAAAAGGGTCTGAGG
HIV F 2LTR	S	AACTAGGGAACCCACTGCTTAAG
HIV R 2LTR	AS	TTGTCTTCGTTGGGAGTGAATTAG
T-GFP	S	CACCATGGTGAGCAAGGGCGAGG
GFP R	AS	CTCGAGTTACTTGTACAGCTCGTCCATG
o- A1	S	TGGGCATGGGTCAGAAGGAT
o- A3	AS	CGGCCAGAGGCGTACAGGGA

2.6.16 Primer usage

Plasmid Name	Usage	Sense	Anti-sense
pLgatewayR5IRESYFP	sequencing/cloning	EG4	EG5
pLgatewayR5IRESYFP	QC mutation	EG22 EG46	EG21 EG47
pLgatewayR7IRESYFP	sequencing/cloning	EG6	EG7
pLgatewayR7IRESYFP	QC mutation	EG11	EG12
pLgatewayR9IRESYFP	sequencing/cloning	EG17	EG18
pLgatewayR9IRESYFP	QC mutation	EG23	EG24
pLgatewayR11IRESYFP	sequencing/cloning	EG15	EG16
pLgatewayR11IRESYFP	QC mutation	EG25	EG26

α-trim1 siRNA plasmid	sequencing	EG13	EG14
pHIT60	sequencing	EG27	EG28
pLgatewayTva800IRESYFP	sequencing	EG68 EG70	EG41 EG42
		EG72 EG74	EG45 EG67
		EG79 EG81	EG69 EG71
		EG91 EG92	EG73 EG80
		EG85 EG86	EG82 EG83
		TS221	EG84 EG90
		TS222	GFP-R
		EG91 EG92	
		T-GFP	
pLgatewayTva800IRESYFP	qPCR	EG55	EG56
pLgatewayTva800IRESYFP	QC mutation	EG39 EG43	EG40 EG44
		EG96	EG97
pLgatewayTva800IRESYFP	HA tag insertion	EG48	EG49
pLgatewayTva800IRESYFP	fragment cloning	EG88	EG89
pLgatewayTva950IRESYFP	sequencing	TS221	TS223
pLgatewayTva950IRESYFP	Fragment cloning	EG88	EG89
pLgatewayp56lckIRESYFP	cloning/sequencing	EG93 EG95	EG94
	CXCR4 detection	EG57	EG58
	CD4 detection	T-CD4	CD4 R
	CD4 sequencing	T-CD4	CD4 R EG54
	QPCR actin	o-A1	o-A2
CSGW	ss qPCR (early)	HIV eF	HIV eR

2.7 Calculations

The standard deviation of the mean (sdm) and standard error of the mean (sem), where used, were calculated using Microsoft Excel.

Chapter 3

Routes of Entry Via Tva800 and Tva950

Lv1 is the activity of a non-human primate factor that restricts HIV-1 (Cowan et al. 2002), and Ref1 is the activity found in human cells that blocks replication of N-MLV (Towers et al. 2000). In early 2004, Trim5α was reported to be the cellular factor responsible for both Lv1 and Ref1 restriction. It was discovered by expressing genes from a rhesus macaque gene library in a population of HeLa cells. The gene that rendered the HeLa cells resistant to HIV-1 infection was selected and cloned, and found to encode Trim5α (Stremlau et al. 2004). Primate variants of Trim5α affect different restriction activities; human Trim5α restricts N-MLV but not HIV-1, but rhesus macaque Trim5α can restrict HIV-1 and N-MLV (Stremlau et al. 2004; Yap et al. 2004).

During these experiments, the HIV-1 virions were pseudotyped with VSV-G protein, which directs the virus into the cell via clathrin-mediated endocytosis. Viruses enter the endocytic pathway, and the fusion reaction of the viral and cellular membranes is triggered by the decreasing pH of the endosomes (Aiken 1997; Sun et al. 2005). Narayan et al. had reported in 2003 that the ASLV envelope could direct a virus into the cell by two different routes depending on which of its two cognate receptors, Tva800 or Tva950, were used (Narayan et al. 2003). These two receptors are formed of identical viral binding domains but are attached to the cell surface in different ways. Tva800 is a GPI-anchored protein, whereas Tva950 has a single membrane-spanning domain. These different attachments mean that the receptors reside in different regions of the cell membrane. The plasma membrane of the cell is not uniform, but composed of different microdomains (Harder and Simons 1997). These microdomains have varying localised concentrations of different phospholipids, fatty acids and membrane proteins. GPI-anchored proteins tend to be found in lipid rafts, whereas proteins with trans-membrane spanning regions are usually excluded from these regions. This was found experimentally to be true for Tva800 and Tva950 (Narayan et al. 2003).

Unlike the fusion reaction subsequent to HIV binding of CD4 which occurs at the cell surface, fusion mediated by ASLV Env and Tva800 or Tva950 requires a low pH step, and both receptors must therefore direct the virus through the endosomal pathway, in which it will experience an increasingly acidic environment. The initiation of fusion occurs in a two-step fashion, unique to ASLV Env. The virus must bind to the receptor, and be taken up into endosomes. Binding of the virus triggers a conformational change in Env that exposes the fusion peptide, which is then inserted into the cellular membrane. Env is now sensitised to low pH, and exposure in the endosomes will complete the reaction, resulting in fusion of viral and cellular membranes. Preventing the acidification of the endosomes by a lysosomotropic agent, for example NH_4Cl, blocks fusion, as the viral envelope protein cannot undergo a conformation change necessary to complete fusion, and the virus will be unable to enter the cell. Lysosomotropic agents are weak bases that can diffuse across membranes. Once in an acidic environment they rapidly become protonated, neutralising the drop in endosomal pH. Maintaining this block for extended periods of time renders viruses that have bound to Tva950 non-viable. Viruses that have bound to Tva800, however, remain infectious for up to 6 hours in the presence of NH_4Cl and can be released to continue down the infectious pathway by simply lowering the concentration to <10mM. The disparity between these two results has been attributed to the difference in membrane attachment between the two receptors, and how these affect the endosomal compartments the virus-receptor complex travels through subsequent to binding (Narayan et al. 2003).

A similar case was reported by Keller et al. (Keller et al. 1992), in which one viral binding domain was attached by two different methods to the plasma membrane. The wild-type CD4 has a single transmembrane spanning domain, but by fusing the extracellular viral ligand-binding domain of CD4 to the C-terminus of decay-accelerating factor, DAF, which mediates transferral to a GPI anchor, a GPI-anchored version was created. The kinetics of uptake and the endocytic pathways embarked on via this receptor, CD4-DAF, and the wild-type form, CD4-TM, were investigated. CD4-TM was found to cluster into pits and was taken up by clathrin-mediated endocytosis, whereas CD4-DAF was taken up by a clathrin-independent pathway from microinvaginations at the cell surface at a slower rate of about 32% that mediated by CD4-TM. This corresponds well with the 2.3-fold higher rate of

endocytosis for Tva950 (transmembrane) relative to Tva800 (GPI-anchored), calculated by Lim et al. (Lim et al. 2004), although it was not directly shown that Tva800 or Tva950 is exclusively associated with either pathway.

Another difference between the two potential CD4 pathways was revealed by incubation with the weak base, primaquine. Addition of primaquine to cells neutralises endosomal pH, and inhibits recycling of receptors back to the cell surface resulting in intracellular accumulation (van Weert et al. 2000). The intracellular pool of CD4-TM almost doubled when primaquine was added, whereas intracellular CD4-DAF levels did not change. This indicates that the endocytic pathways taken by the receptors lead to compartments with different sensitivities to pH neutralisation by primaquine. Both receptors normally recycle back to the cell surface. Gold particles incubated with either CD4-TM or CD4-DAF eventually associated with multivesicular bodies, suggesting that the pathways converge (Keller et al. 1992).

The similarities between this example and the Tva800/950 pathways are evident. Both Tva800 and Tva950 can ultimately direct the virus towards a successful infection. However, as for CD4-DAF and CD4-TM, the routes that are taken after binding to Tva800 or Tva950 prior to entry of the virus into the cytoplasm are thought to differ. Both routes are blocked by the addition of a dominant negative form of dynamin, a component of endocytosis mediated by both clathrin and caveolae (Mothes *et al.* 2000).

All recent work with Trim5α and Fv1 was carried out using VSV-G pseudotyped virions. VSV-G directs virions into the cell via clathrin-mediated endocytosis (Aiken 1997). While certain events that occur between a retrovirus binding to a cell-surface receptor and integration of the proviral DNA into the host cell genome are described to a certain extent, many aspects remain unclear. For example the precise sequence, location, and extent of events that occur on the route the virus takes between the site of entry into the cell cytoplasm and the cytoplasmic/nuclear boundary remain undefined. How does the site of fusion (at the cell surface or out of an endocytic compartment) and therefore the site of viral entry into the cytoplasm affect the inhibitory factors and other defence mechanism the cell mounts to try and prevent retroviral infection? What events are concurrent with trafficking to the nucleus?

How do these vary between different retroviruses? A system that allows the user to vary entry events and conditions would aid clarification of some of these issues.

At the commencement of these studies Fv1 was known to be located to the cytoplasm, but Trim5α was less well locally defined (Yap and Stoye 2003). It was of interest to see if taking a different route to the cytoplasm could allow the virus time to mature past a point of susceptibility to restriction, or bypass the restriction altogether. If Tva800 and Tva950 do send the virus on significantly different routes of entry into the cell, then they could be useful in analysing whether restriction by Trim5α and Fv1 has a route-of-entry component that can be circumvented. For this, at least one pathway would need to be distinct from that mediated by VSV-G, with different biochemical or kinetic parameters. The receptors were therefore cloned and the routes taken by virions subsequent to binding to Tva800 or Tva950 were investigated.

Results

3.1 *Mus dunni* cells expressing Tva800 or Tva950 support infection and replication by NB-MLV cores pseudotyped with ASLV envelope

Mus dunni tail fibroblast cells are not known to harbour any endogenous restriction factors, and they support replication of MLV. They were therefore used as the basis for these studies. To ensure that they were permissive to viruses carrying ASLV envelope, *Mus dunni* cell lines expressing Tva800 or Tva950 were prepared. The parental cell line was transduced with the vector pLgateway800IRESG418 or pLgateway950IRESG418, which encode Tva800 or Tva950 respectively, and neomycin resistance (see chapter 2 for all vector maps). Three days after infection daughter cells were replated, and single cell clones were selected by addition of G418 to the medium for two weeks and thereafter. $5x10^4$ *Mus dunni* cells stably expressing Tva800 (d800), Tva950 (d950), or the parental cell line, were infected with between 2-250μl virus comprising NB-MLV core, ASLV envelope and carrying vector encoding eGFP. Three days later cells were harvested, fixed and prepared for analysis by FACS.

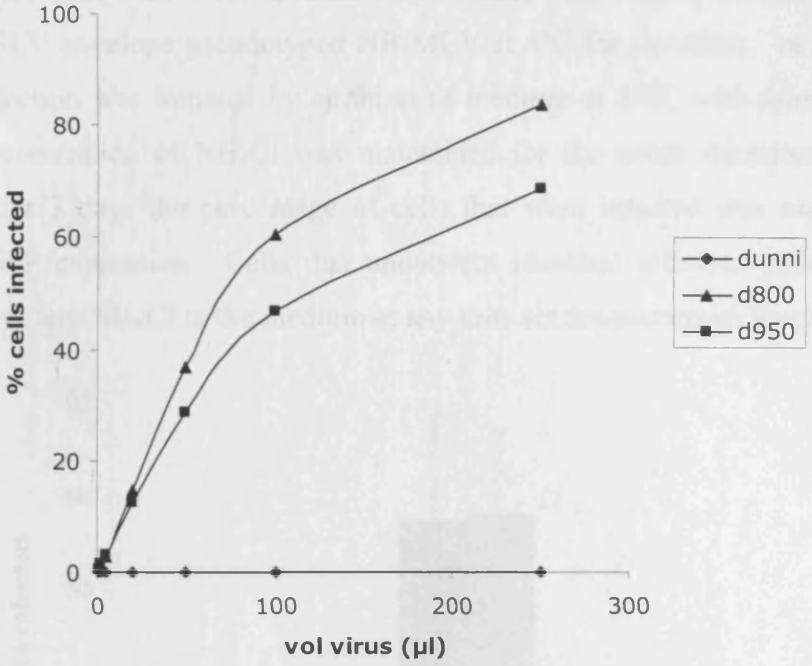


Fig. 3.1 *Mus dunni* cells expressing Tva800 or Tva950 support infection by NB-MLV cores pseudotyped with ASLV envelope. $5x10^4$ d800, d950 and *Mus dunni* cells were transduced with increasing volumes (1-250µl) of ASLV Env pseudotyped NB-MLV. The percentage of cells successfully infected was assayed by FACS for GFP expression 3 days later. Unfortunately no standard cell line was readily available to titrate the virus. The parental *Mus dunni* cell line was completely uninfectable by ASLV Env pseudotyped virus, but expression of either Tva800 or Tva950 rendered the cells permissive. The experiment was repeated twice with the same clones with identical results, so these clones were named d800 and d950 and used throughout. The d800 cell line was further used subsequently to standardise stocks of ASLV env pseudotyped virus.

The results of this experiment presented in figure 3.1, show that the parental cell line is totally non-permissive to ASLV Env pseudotyped MLV cores, but introduction of Tva800 or Tva950 is sufficient to render the cells susceptible to infection. Thus, these cell lines can be used as a basis for further experiments. HIV-1 cores pseudotyped with ASLV Env were also able to infect d800 and d950 cells (not shown).

3.2 Viruses bound to Tva800 receptors remain infectious for 6 hours if entry is blocked

To ensure that these receptors functioned in *Mus dunni* cells, as previously described (Narayan et al. 2003; Narayan and Young 2004), the effects of NH_4Cl on viral entry were investigated. $5x10^4$ d800 or d950 cells were plated and allowed to settle. 24

hours later cells were incubated for 1 hour with 40mM NH_4Cl, prior to binding of ASLV envelope pseudotyped NB-MLV at 4°C for one hour. In control experiments infection was initiated by addition of medium at 37°C with 40mM NH_4Cl, and this concentration of NH_4Cl was maintained for the entire duration of the experiment. After 3 days the percentage of cells that were infected was assayed by FACS for eGFP expression. Cells that underwent identical infection procedures but did not have any NH_4Cl in the medium at any time set the maximum level of infection.

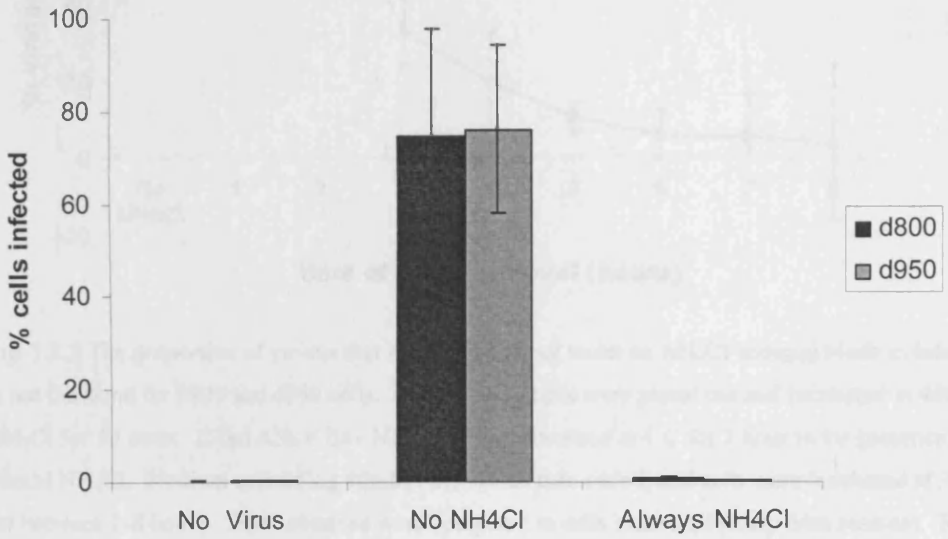


Fig 3.2.1 ASLV Env pseudotyped viruses are unable to infect cells succesfully in the presence of 40mM NH_4Cl. d800 or d950 cells were plated, and 24 hours later cells were incubated in medium containing 40mM NH_4Cl for 30 minutes. Cells were transduced with 200µl ASLV Env pseudotyped NB-MLV in 1mL medium containing 40mM NH_4Cl. Results shown are combined from 3 separate experiments, and error bars show sem.

Data in figure 3.2.1 show that when NH_4Cl is maintained in the cell medium for the duration of the experiment viral infection is entirely blocked, there is no difference in infection levels (i.e. GFP expression) between cells infected with virus under an NH_4Cl block and cells that do not have any virus on them at all.

In a second set of experiments, cells were incubated with virus and infection was initiated as described above. At preset timepoints after infection of between 1 and 8 hours the NH_4Cl-containing medium was removed, and replaced with DMEM only, potentially releasing virions from the effects of a block caused by inhibition of acidification of the endosomes.

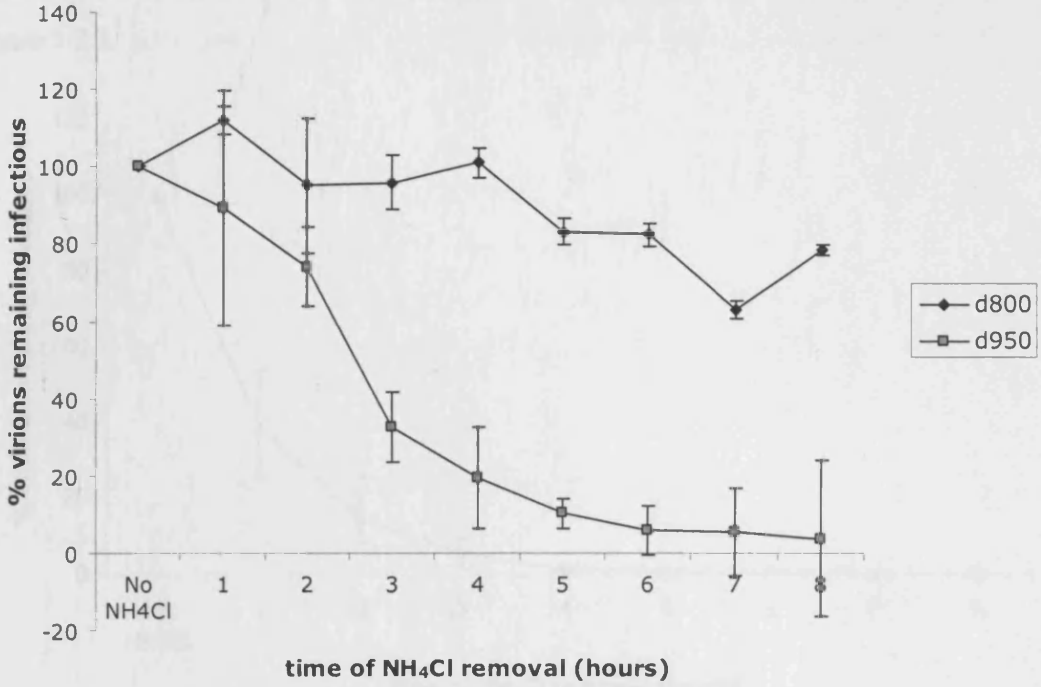


Fig. 3.2.2 The proportion of virions that remain infectious under an NH_4Cl-induced block to infection is not the same for d800 and d950 cells. d800 or d950 cells were plated out and incubated in 40mM NH_4Cl for 30 mins. 250µl ASLV Env NB-MLV was adsorbed at 4°C for 1 hour in the presence of 40mM NH_4Cl. Medium containing 40mM NH_4Cl was then added, and cells were incubated at 37° C for between 1-8 hours. Titres obtained were compared to cells incubated solely with medium. Results are combined from 3 separate experiments, and error bars show sdm.

Figure 3.2.2 shows the effect of removing the NH_4Cl block from d800 and d950 cells between 1-8 hours after infection. The percentage of GFP-expressing cells is an indication of the percentage of virions remaining viable at the time of NH_4Cl removal. Removal of this block at set time points after infection reveals the length of time that infection can be arrested while viral particles still remain viable. In d950 cells infectivity decreases rapidly with incubation under NH_4Cl, showing that the virus is no longer able to complete the infectious cycle. However, when virions have bound to Tva800, >80% remained infectious even when the infectious pathway was blocked for 8 hours.

In order to see how the d800 and d950 infectivity periods compare with another receptor also blocked by NH_4Cl treatment, an identical experiment was carried out as

described above with NB-MLV pseudotyped with VSV-G. The results are shown in figure 3.2.3.

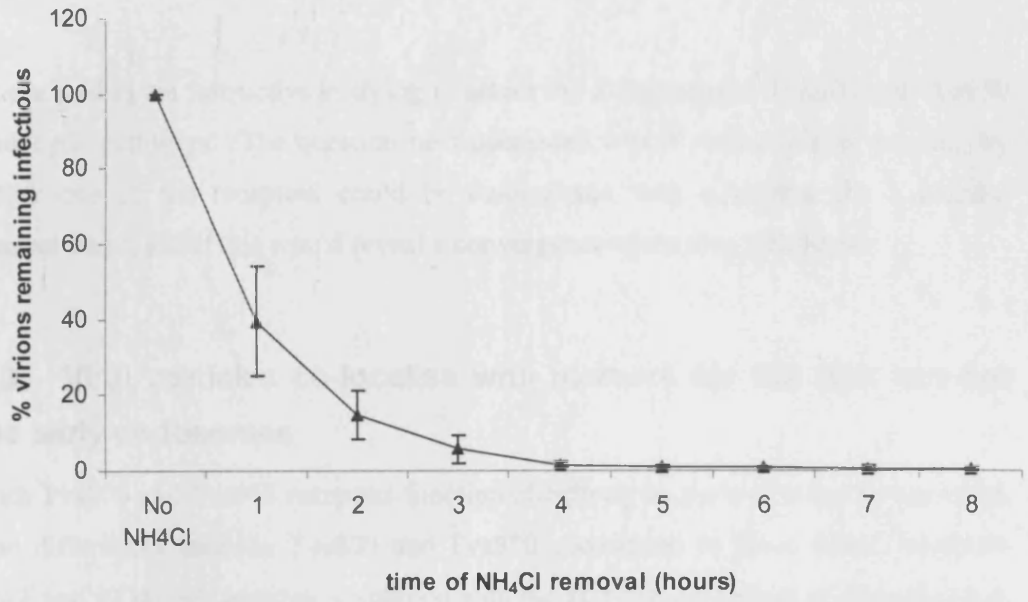


Fig. 3.2.3 Proportion of virions bound via VSV-G envelope that are able to complete an infectious cycle after being blocked by NH_4Cl for varying times. The experiment was carried out as described in the legend to figure 3.2.2. Results shown are combined from 3 experiments, and error bars show sdm.

As can be seen, the decrease in infectivity is even more dramatic than that for d950 cells, with fewer than 15% of virions remaining infectious after only 2 hours NH_4Cl treatment. This confirms that the stability seen with ASLV pseudotyped virions bound to Tva800 is indeed exceptional. The instability seen with d950 cells could be either because the viral particle itself is not stable under conditions where the acidification of subcellular organelles is blocked, or because the virus-receptor complex in the compartment it is trapped in is somehow unstable. Over 80% of viruses that have bound to d800 cells remain viable when they are trapped for 8 hours, suggesting that the degradation under blockage experienced by viruses bound to Tva950 is not related to instability of the virus per se, but to the cellular environment in which it is bound and held.

The viral binding domains of the receptors Tva800 and Tva950 (contained entirely within the first 102 amino acids) are identical, therefore the difference between the two pathways must relate to their attachment to the cell membrane, the

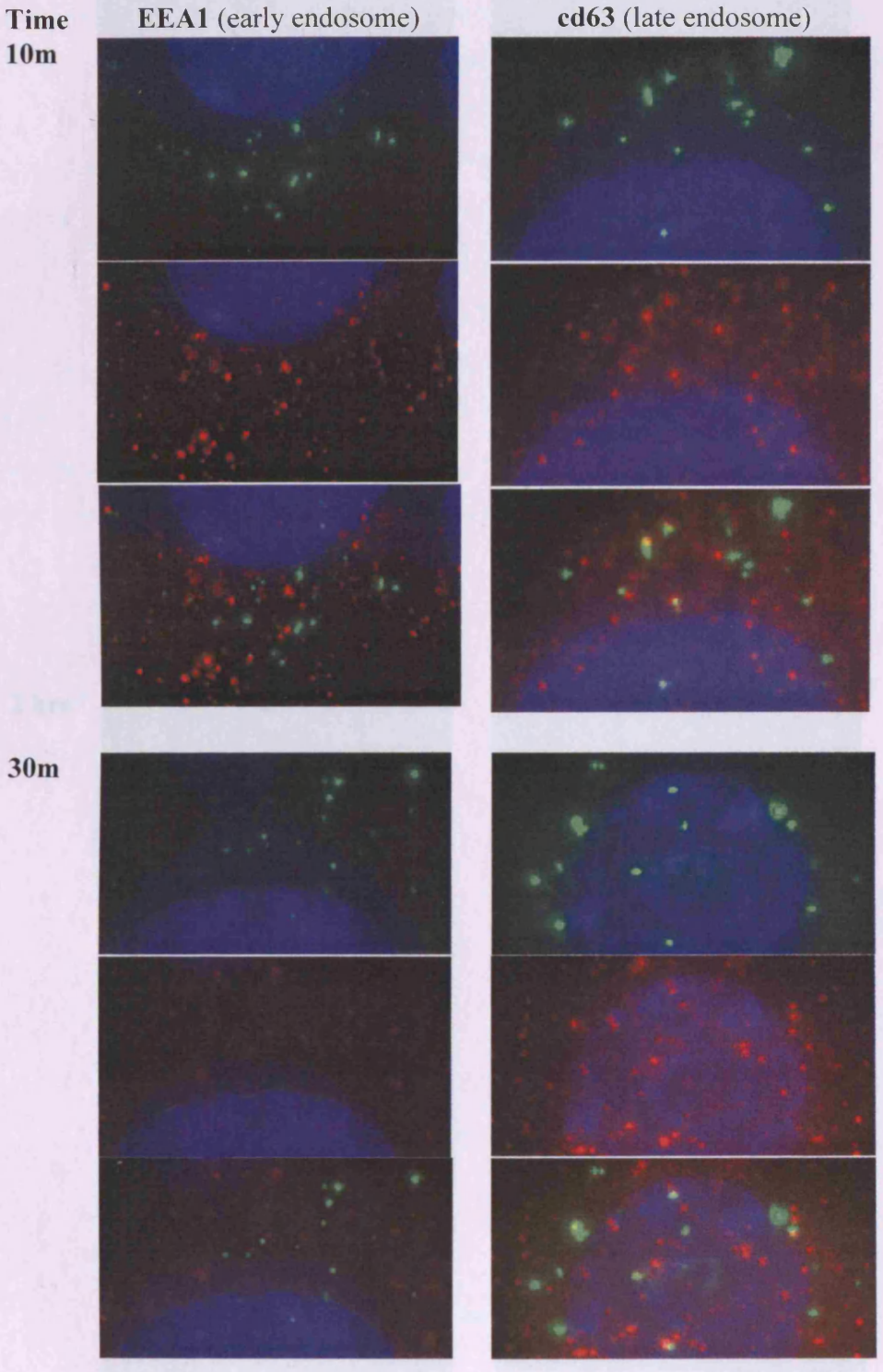
microenvironment of the membrane in which the receptors are found, and the way these two factors affect the route by which virions binding to the receptors enter the cell.

These studies are instructive in trying to assess the differences in Tva800 and Tva950 endocytic pathways. The question next addressed was if viral particles entering by either one of the receptors could be co-localised with a marker for a cellular compartment, and if this would reveal a convergence of the two pathways.

3.3 Viral particles co-localise with markers for the late, but not the early endosomes

Both Tva800 and Tva950 receptors function effectively as ports of entry for the virus. The differences between Tva800 and Tva950 correspond to those found for CD4-DAF and CD4-TM, and this, combined with the fact that regardless of receptor type, the ASLV envelope needs low pH to trigger the fusion reaction, suggests that at some point the pathways taken by the virus after entry through one receptor or the other must pass through a low pH compartment, and could conceivably converge. In order to try and ascertain where the block to infection in the presence of NH_4Cl, and potentially where this point of convergence might be, viral particles were made with an NL4.3 (HIV-1) core, ASLV envelope, and GFP-vpr ('green HIV'). GFP-vpr is a fusion protein made of the accessory protein vpr, and GFP, which renders it fluorescent (McDonald et al. 2002). GFP-vpr, like vpr, is taken up in high copy number by viral particles, and usefully remains associated with the viral ribonucleic acid, so viral particles can be tracked by this fluorescence as they move through the cell. The green HIV was concentrated, placed on d800 cells at 4°C, and allowed to bind for an hour in the presence of 40mM NH_4Cl. After one hour the cells were washed and warm (37°C) medium + 40mM NH_4Cl was added, and the cells incubated for 10, 30, 60 or 120 minutes. The infection was halted by removal of medium and addition of 4% formaldehyde in PBS. Cells were then permeabilised and stained red for either early endosomes (anti-EEA1) or late endosomes/ multivesicular bodies (MVB) (anti-lamp3), and visualised under the microscope. At least four pictures were taken for each condition, and one example is shown in figure 3.3.1. Green viral particles (top pictures) were scored as co-localised with a red endosomal marker

(middle pictures) if signals from the two markers overlay each other (bottom pictures), as analysed in Adobe Photoshop.



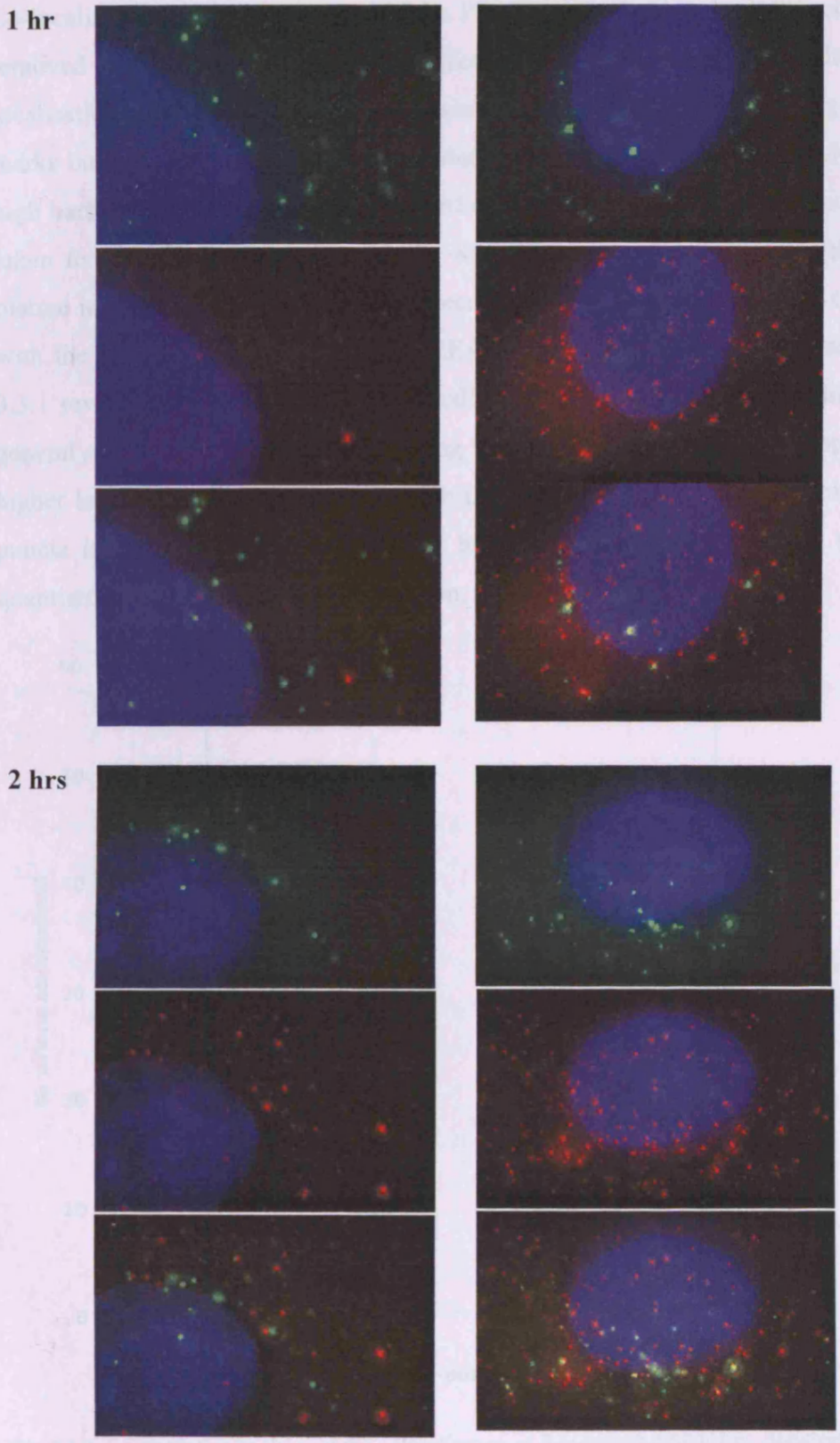


Fig. 3.3.1 Fluorescent ASLV Env pseudotyped virions colocalised with markers for the late endosome/MVB in Tva800 cells. $1x10^5$ d800 cells were plated on glass coverslips, and infected at MOI 5 in the presence of 40mM NH_4Cl. After between 10-120 minutes cells were fixed and stained with anti-EEA1 (early endosomal marker) or anti-cd63 (found on the late endosomes and the MVB).

Co-localisation was scored using Adobe Photoshop software; the green channel was removed from pictures, and if a red fluorescent mark found to be underneath co-localisation was confirmed. A rough assessement can be made by looking for yellow marks but this does not always reveal faint red fluorescence, and can be skewed by a high background of red that is non-specific staining. Between 4 and 6 pictures were taken for each condition, with at least 40 virions per condition, although only one picture is shown as an example. The green virions were only rarely seen co-localised with the early endosomes stained by EEA1, so that the left-hand column of figure 3.3.1 reveals few yellow puncta. For cd63, however, while the red staining did in general appear to be higher than staining with EEA1, there was a disproportionately higher level of co-localisation seen with the green virions, resulting in many yellow puncta in these pictures. All pictures taken were scored in this way to obtain a quantitative assessment of co-localisation, shown in figure 3.3.2.

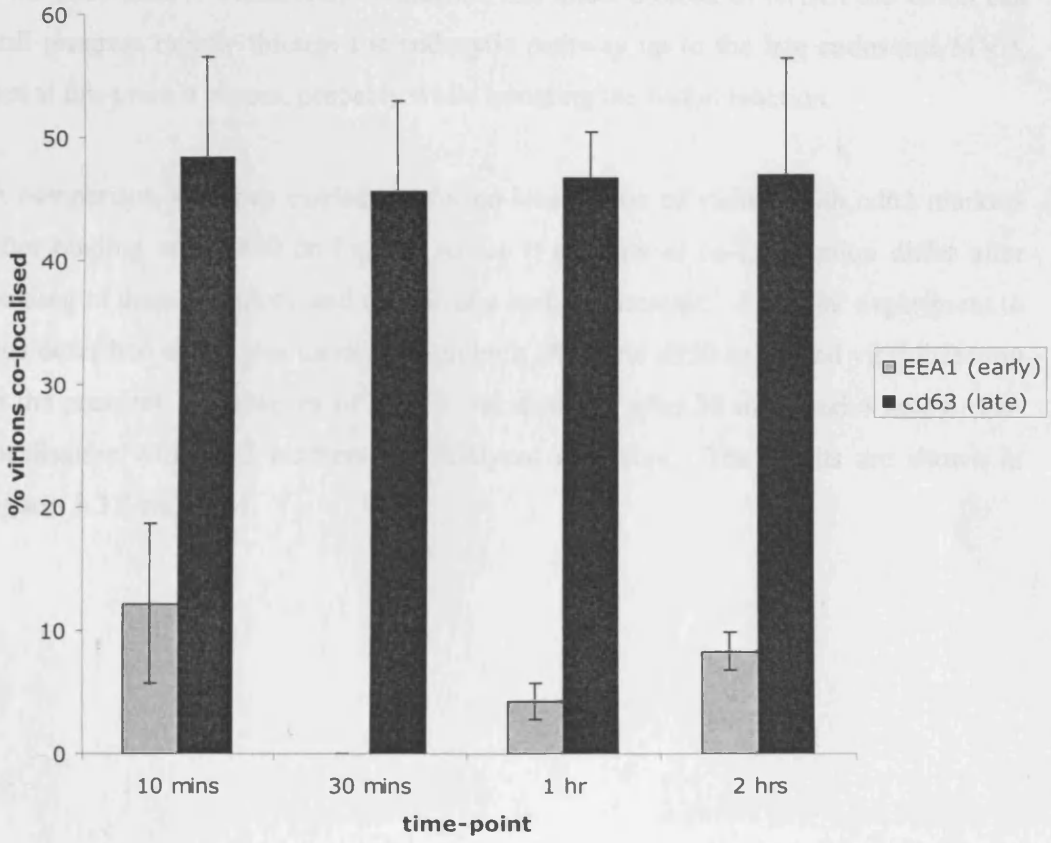


Fig. 3.3.2 A quantitative analysis of the colocalisation of fluorescent ASLV Env pseudotyped virions with markers for the late endosome/MVB in Tva800 cells. Cells were scored for the percentage of virions found colocalised with these markers, i.e. the red and green markers significantly overlay when analysed with Adobe Photoshop. Error bars represent the standard deviation of the mean number of virions colocalised across the 4-6 pictures taken for each sample.

The time at which the highest percentage of virions were found to be co-localised with markers for EEA1 (found on the early endosome) was when virions had been allowed to infect cells for a total of 10 minutes. Even then, only 12% of virions co-localised. This does not mean that the virion does not pass through the early endosome, rather that if it does, it must pass through very rapidly and not be held there for any significant length of time. The number of virions found co-localised with cd63 was consistently higher, between 45 and 50% for every time period analysed up to 2 hours.

The experimentally determined pH at which ASLV-A Env mediated fusion out of the endocytic pathway occurs is <pH5.5 (Mothes et al. 2000). The pH of early endosomes is usually above this, between 5.9-6.0, not dropping to below 5.5 until the late endosome is forming, indicating ASLV-A exit at these later stages. Together with these data, it would seem to indicate that under a block of NH_4Cl the virion can still progress rapidly through the endocytic pathway up to the late endosome/MVB, but at this point it pauses, probably while initiating the fusion reaction.

A comparison was then carried out for co-localisation of virions with cd63 markers after binding to Tva800 or Tva950, to see if patterns of co-localisation differ after binding to these receptors, and to look at a longer timescale. A similar experiment to that described above was carried out on both d800 and d950 cells, and viral infection in the presence and absence of NH_4Cl was analysed after 30 mins and 4 hours. Co-localisation with cd63 markers was analysed as before. The results are shown in figures 3.3.3 and 3.3.4.

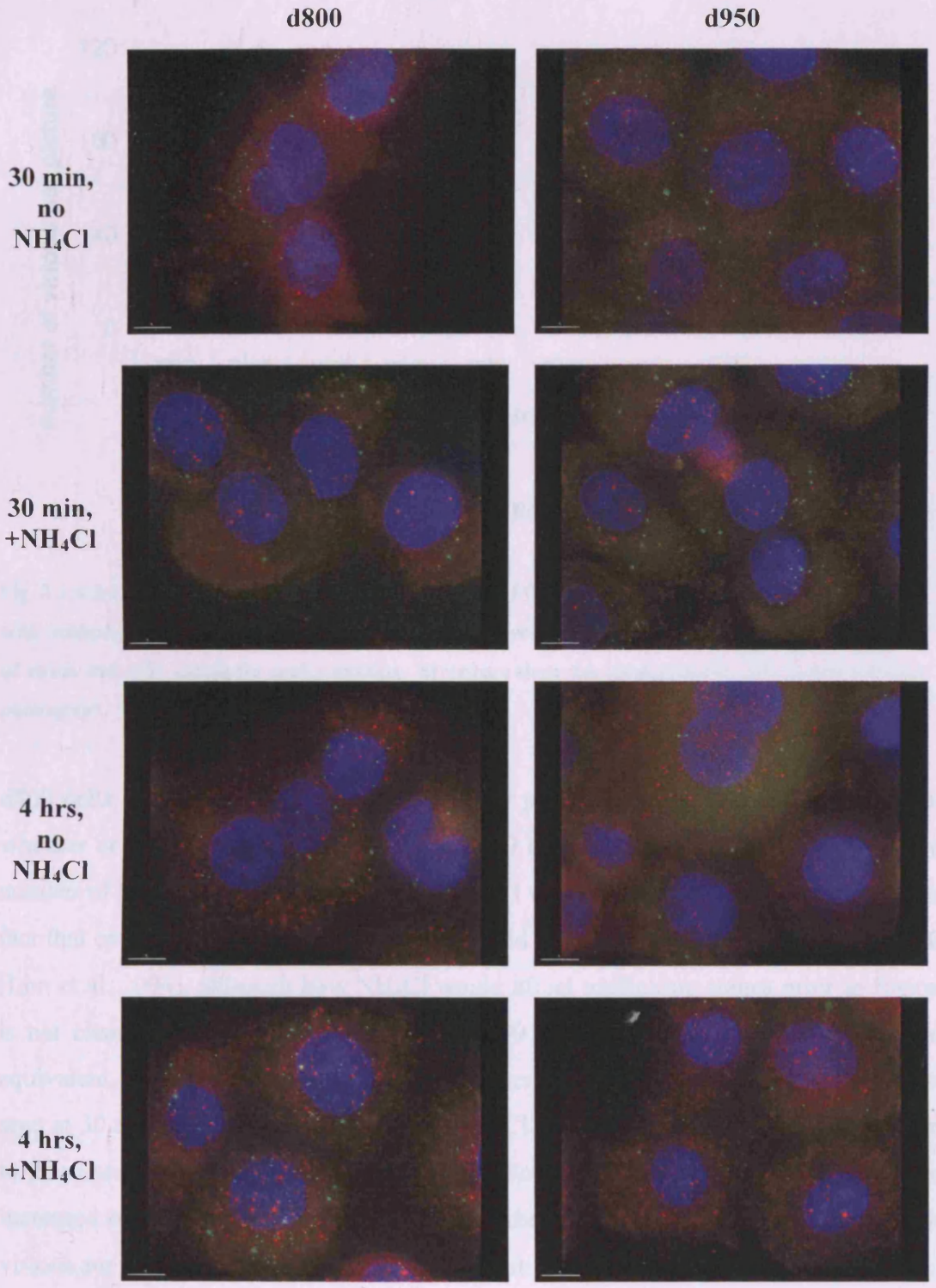


Fig. 3.3.3 Co-localisation of fluorescent ASLV Env pseudotyped virions with markers for cd63 at 30 mins and 4 hours. cd63 is found on late endosomes and in multivesicular bodies. The experiment was performed as described in the legend to figure 3.3.1 for d800 and d950 cells, except that no staining of EEA1 was done.

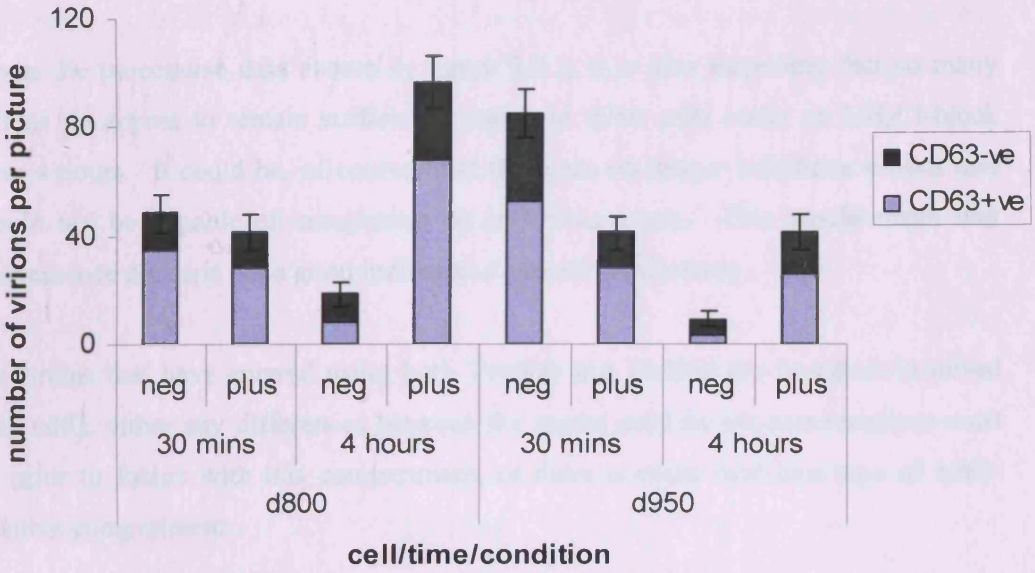


Fig. 3.3.4 A quantitative analysis of the co-localisation of fluorescent ASLV Env pseudotyped virions with markers for cd63 at 30 mins and 4 hours. Pictures were randomised and scored blind for overlap of virion and cd63 signals for each condition. Error bars show the standard error of the count for each photograph.

d800 cells had on average over 50 virions per picture after 30 minutes of infection, whether or not NH_4Cl was present. The d950 cells had almost double this average number of virions after 30 minutes when NH_4Cl was not present. This may reflect the fact that entry via Tva950 is on average 2.6-fold faster compared to entry via Tva800 (Lim et al. 2004), although how NH_4Cl would affect trafficking stages prior to fusion is not clear. After 4 hours the levels in d800 and d950 NH_4Cl-negative cells are equivalent, and for both cell types total numbers of virions are under 50% of those seen at 30 minutes. For d950 cells under NH_4Cl, the levels of virions are comparable to those seen after 30 minutes under NH_4Cl. For d800 cells, however, the levels are increased by ~2.5-fold, which implies that in the extra 3½ hours greater numbers of virions are able to both enter the cell (and so not be washed off in steps preparatory to binding antibody) and remain intact and infectious in this time. In retrospect 30 minutes probably was not sufficient time for a first time-point. Virus was bound at 4°C for an hour, and although at time zero pre-warmed medium is added, it can still take as long as 20 minutes for a 12-well dish to properly equilibrate at 37°C, which

does not really allow the virions sufficient time to initiate entry before the experiment was halted.

Given the timecourse data shown in figure 3.2.2, it is also surprising that so many virions do appear to remain sufficiently intact in d950 cells under an NH_4Cl-block after 4 hours. It could be, of course, that these are no longer infectious virions and would not be capable of completing an infectious cycle. This would mean that fluorescence per se is not a good indicator of potential infectivity.

As virions that have entered using both Tva800 and Tva950 are found co-localised with cd63, either any differences between the routes used by the two receptors must be prior to fusion with this compartment, or there is more than one type of cd63-positive compartment.

3.4 Inhibition of Rab5, but not Rab7, decreases entry via Tva800 and Tva950

Having established that the pathways of entry of the virus subsequent to binding and entry via Tva800 or Tva950 converge on the late endosome, the question then arises as to the differences between Tva800 and Tva950 pathways before the late endosome. If pathways subsequent to binding are different, would alteration of cellular conditions affect the virus as it traffics prior to this point to differentiate the two pathways? To address this question, a set of cellular traffic controllers, the Rab proteins, were considered. Rabs are small GTPases that are found at several points in the vesicle trafficking pathways of the cell. Crucially, one Rab protein is characteristically associated with a subset of endocytic vesicles, or a single stage of a pathway. For example, Rab5 is found on endocytic vesicles prior to fusion with early endosomes and on early endosomes themselves, and Rab7 is involved in late endosome maturation and lysosome biogenesis. Rab9 controls transport between the late endosome and the golgi, and Rabs 4 & 11 are involved in recycling back to the plasma membrane (see figure 1.9.2 for an illustration of these pathways).

Functional Rabs exchange GDP for GTP as part of their cycle from inactive to active, respectively. Dominant negative forms of these Rab proteins that preferentially bind

GDP have long been used to study the effects of their inhibition (Sieczkarski and Whittaker 2002a). Due to GDP binding, the dominant negative Rab is permanently switched off. When a dominant negative version of a Rab protein is over-expressed in a cell it swamps the wild-type functional Rab, down-modulating its activity.

To assay the effects of expression of an exogenous factor on a large population of cells swiftly, an assay was developed that allows a non-clonal population of cells expressing this exogenous factor to be directly compared with factor-naïve cells in a single well. The use of single cell clones can sometimes lead to false conclusions being drawn, if a single cell colony is picked that is genetically positive for the exogenous factor, but shows no effects. The use of a non-clonal cell population in which there is a range of expression levels suppresses rare effects and emphasises general trends (Bock et al. 2000; Bishop 2001). The gene of interest is cloned into the plasmid pLgateway**X**IRESYFP. The gene of interest is inserted at the X, and expression is driven by the MLV LTR. YFP expression is driven from an IRES. Cells transduced with this vector that are positive for YFP expression also express the inserted gene of interest. Three days after the initial transduction cells are further infected with the challenge virus, which carries EGFP vector. After a further three days, four populations of cells can be seen on FACS analysis. Double negative cells are infected with neither the factor under consideration nor challenge virus, cells expressing EYFP only have not been infected with the challenge virus, those cells expressing EGFP only are factor negative but challenge virus positive, and double positive cells for EYFP and EGFP express factor and were infected by the challenge virus. The proportion of cells that expressed the gene of interest that were successfully infected can be compared to the proportion that were infected in the population that did not express any extra factor. Comparison of proportions of these populations of cells allows the effects of exogenous factors on viral replication to be determined (see section 2.2.2 for a worked example).

This assay was used to assess the effects of the dominant negative Rab proteins on viral replication. Primers to amplify the ORFs of Rabs 5, 7, 9 and 11 were designed from published sequences (GeneIDs 5868, 7879, 9367 and 8766, respectively) and amplified out of a HeLa library. Dominant negatives Rab5 S34N, Rab7 T22N, Rab9 S21N and Rab11 S25N have been designed and characterised previously (Riederer et

al. 1994; Stenmark et al. 1994; Ullrich et al. 1996; Vidricaire and Tremblay 2005). These mutations were introduced into the plasmids by QuikChange mutagenesis. Challenge virus titres on cells expressing the dominant negative Rab proteins (and EYFP) were compared with titres in Rab DN null cells. The results are shown in figure 3.4. A fold-difference of 1 indicates that titres of challenge virus were not altered and <0.7 indicates attenuation of viral titre through inhibition of viral replication by the expression of the dominant negative Rab. Titres are not normally completely reduced by dominant negative Rabs as a significant population of wild-type, functional Rab protein remains in the cell.

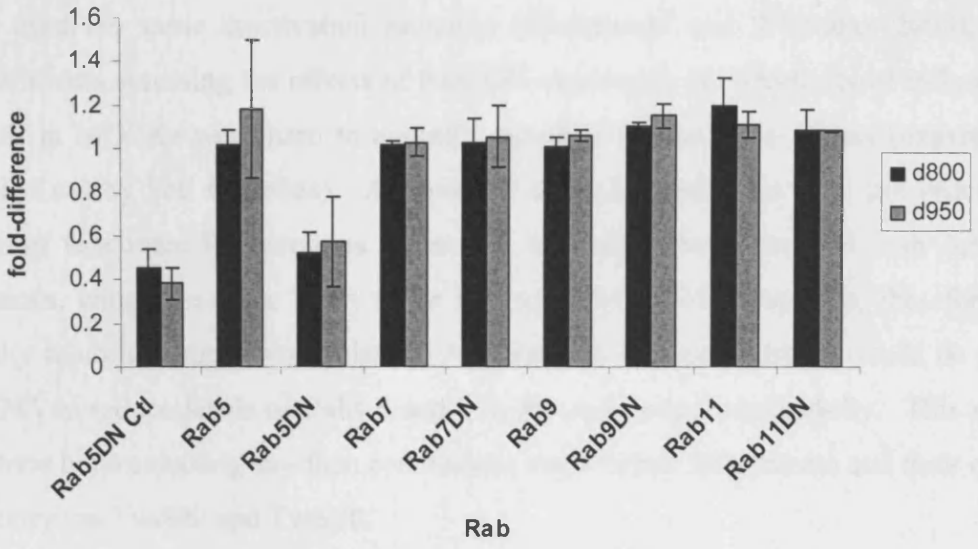


Fig 3.4 Inhibition of Rab5 alone decreases titres of ASLV Env pseudotyped NB-MLV. $5x10^4$ d800 or d950 cells were plated and transduced with Rab vector at MOI 1. 2 days later cells were split, and after 1 further day challenged with ASLV Env pseudotyped NB-MLV. The control experiment for Rab5DN carried out using VSV-G pseudotyped NB-MLV is also shown (Rab5DN Ctl). Inhibition was monitored by calculating the ratio of the percentage of cells expressing Rab compared to the percentage of negative cells, for each well. Results shown are combined from two separate experiments, and error bars show sdm.

As can be seen from figure 3.4, only Rab5DN decreased viral titres to any extent, and the effect was identical for both Tva800 and Tva950 cells. As Rab5 is involved in regulating traffic from the plasma membrane to early endosomes this would indicate that part of this pathway is crucial for viral entry via both Tva800 and Tva950. After the virus has been endocytosed, Rab5 is probably therefore involved in directing the

endocytic vesicle to the early endosome, or in the fusion reaction between the endocytic vesicle and the early endosome. Given that the microscopy data presented in figs 3.3.1 to 3.3.4 earlier would indicate that viruses colocalise substantially with markers for the late endosome/MVB, and that Rab7 is substantially involved in trafficking to, and is a marker for, the late endosome, it is surprising that no inhibitory effect is seen with Rab7DN. Fusion of the virion via ASLV Env out of the endosomal pathway is triggered at a pH of below 5.5 (Mothes et al. 2000), which is lower than the pH reached in the early endosome. Control experiments to assess the inhibitory effects of Rab7DN on influenza infection have been carried out by previous groups that used the same inactivation mutation (Sieczkarski and Whittaker 2003). Our experiments assessing the effects of Rab7DN expression on infectivity of influenza A (PR8) in MDCKs were hard to quantify, possibly due to toxic effects (experiments carried out by Seti Grambas). Addition of dominant negatives does not reduce the level of functional Rab proteins in the cell, but rather floods the cell with defective versions, which are more likely to be interacted with. Pathways are, therefore, not totally inhibited, but downregulated. A better way to assess effects would be to use SiRNA to reduce levels of Rabs 5 and 7 in the cells more substantially. This should be done before making any firm conclusions about either Rab protein and their effects on entry via Tva800 and Tva950.

3.5 Route of entry via Tva800 or Tva950 receptor does not affect whether NB-, N- or B-tropic MLV is affected by Fv1 or Trim5α

One reason to investigate the differences between Tva800 and Tva950 was to assess the effects of route of entry on restriction by Fv1 and Trim5α. It could be hypothesised that forcing the virus to take an alternative route into the cell to its usual course could permit it to evade restriction activity. In order to assess whether this could be the case for Tva800 and Tva950, the 2-colour assay was adapted, with d800 and d950 cells described above used as the basis for this assay. The d800 or d950 cells were transduced at MOI ~1 with $Fv1^n$, $Fv1^b$ or Trim5α. This MOI results in approximately 40% of the target cell population becoming YFP positive, and by extension restriction factor positive. Three days later, cells were then challenged with ASLV envelope-pseudotyped NB-, N- or B-tropic MLV with between 2-100µl. Results were analysed by FACS three days later.

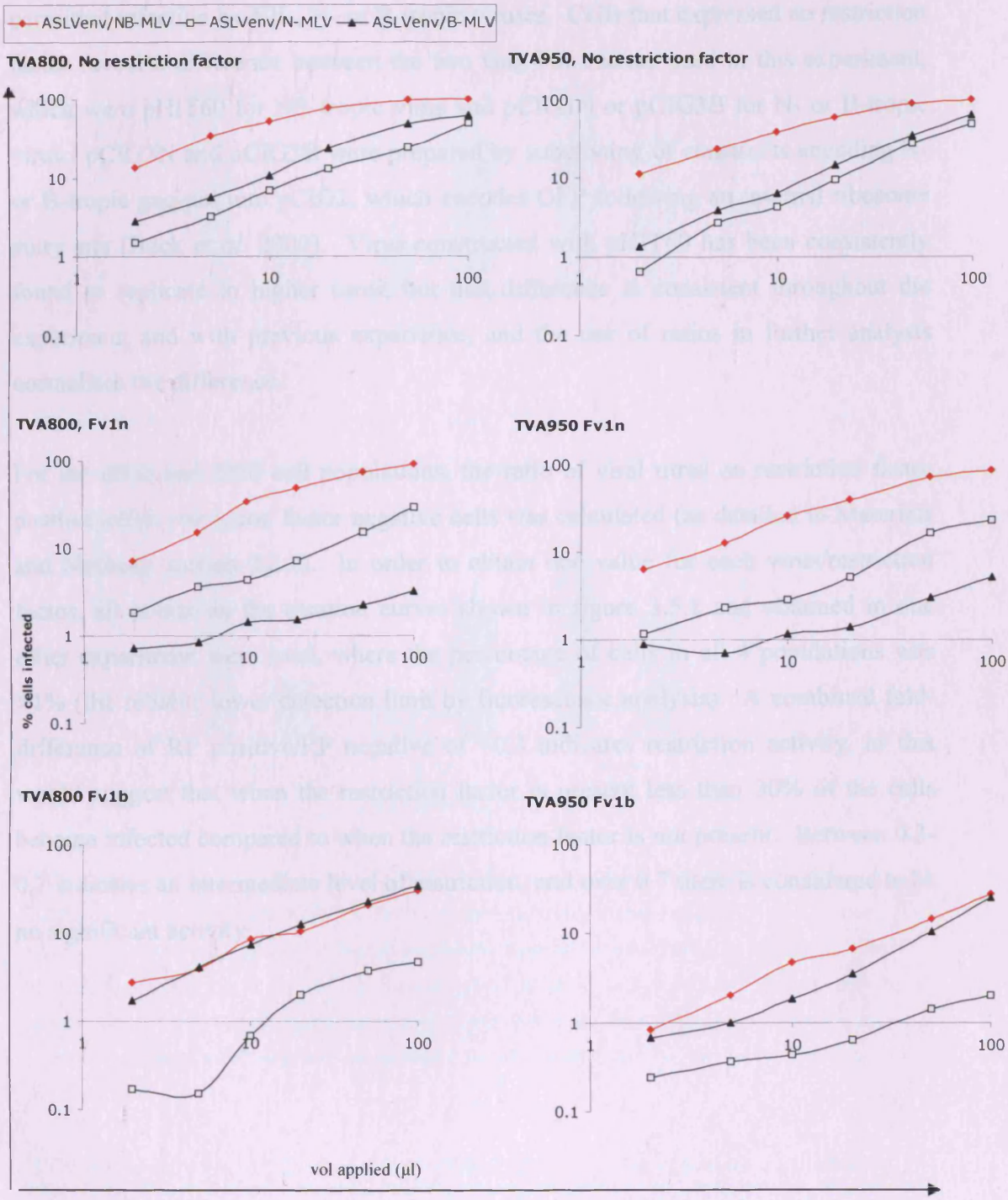


Fig. 3.5.1 Restriction of N-, B- and NB-tropic MLV by Fv1 and Trim5α is not affected by entry via Tva800 or Tva950. d800 and d950 cells were plated out and transduced with $Fv1^n$, $Fv1^b$ or Trim5α. Two days later cells were passaged and challenged with 2-100µl ASLV Env pseudotyped N-, B-, or NB-MLV. The restriction of the virions by the restriction factor was compared in each cell line. The x-axis shows the volume of virus applied, in µl, and the y-axis the percentage of cells infected. One experiment is shown as an example.

Data in fig 3.5.1 show the percentage of cells expressing $Fv1^n$, $Fv1^b$ or Trim5α that permitted infection by NB-, N- or B-tropic viruses. Cells that expressed no restriction factor reveal a difference between the two Gag-Pol vectors used in this experiment, which were pHIT60 for NB-tropic virus and pCIG3N or pCIG3B for N- or B-tropic virus. pCIG3N and pCIG3B were prepared by subcloning of constructs encoding N- or B-tropic gag-pol into pCIG2, which encodes GFP following an internal ribosome entry site (Bock *et al.* 2000). Virus constructed with pHIT60 has been consistently found to replicate to higher titres, but this difference is consistent throughout the experiment and with previous experience, and the use of ratios in further analysis normalises the difference.

For the d800 and d950 cell populations, the ratio of viral titres on restriction factor positive cells: restriction factor negative cells was calculated (as detailed in Materials and Methods section 2.2.2). In order to obtain one value for each virus/restriction factor, all points on the titration curves shown in figure 3.5.1 and obtained in one other experiment were used, where the percentage of cells in all 4 populations was >1% (the reliable lower detection limit by fluorescence analysis). A combined fold-difference of RF positive/RF negative of <0.3 indicates restriction activity, as this would suggest that when the restriction factor is present less than 30% of the cells become infected compared to when the restriction factor is not present. Between 0.3-0.7 indicates an intermediate level of restriction, and over 0.7 there is considered to be no significant activity.

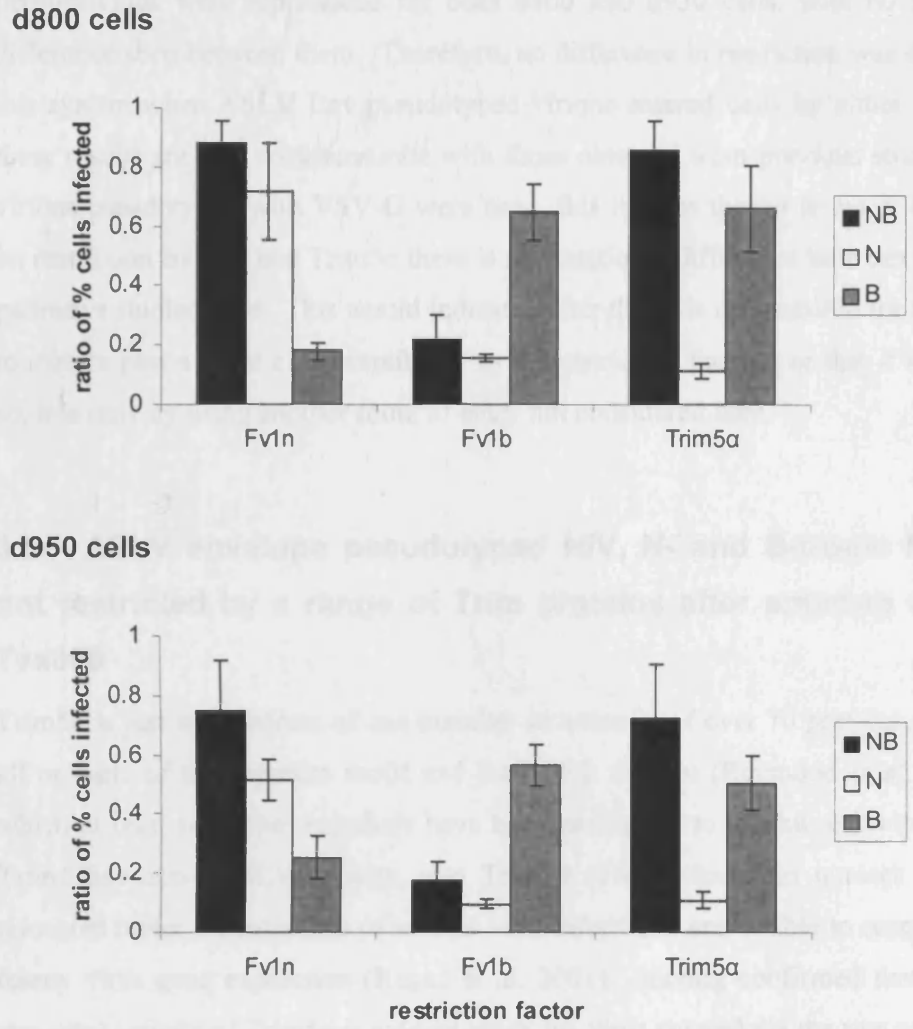


Fig 3.5.2 Restriction of N-, B- and NB-tropic MLV by Fv1 and Trim5α is not affected by entry via Tva800 or Tva950. The experiment was carried out as described in legend to figure 3.5.1. Columns plotted are the proportion of Fv1 or Trim5a positive cells infected compared to negative cells. Results shown are combined from 2 separate experiments, and all points of the titration curves obtained used to generate the above data as long as the percentage of cells in each quadrant (RF and virus-negative, RF-positive virus-negative, RF-negative virus-positive, and RF and virus-positive) was >1%. Error bars represent sdm.

The decreases in viral titres caused by Fv1 or Trim5α were as expected, and generally in accordance with previously reported values. More specifically: $Fv1^n$ or Trim5α did not restrict NB-tropic MLV; $Fv1^b$ and Trim5α restricted N-tropic MLV; and $Fv1^n$ restricted B-tropic MLV. As seen in previous assays, $Fv1^b$ also strongly restricted NB-tropic MLV (Bock et al. 2000). However, all of these restrictions and

permissivities were reproduced for both d800 and d950 cells, with no significant difference seen between them. Therefore, no difference in restriction was detected in this system when ASLV Env pseudotyped virions entered cells by either route. As these results are also commensurate with those obtained from previous studies where virions pseudotyped with VSV-G were used, this implies that in terms of the effects on restriction by Fv1 and Trim5α there is no functional difference between any of the pathways studied here. This would indicate either that it is not possible for the virions to mature past a point of susceptibility to the restriction factors, or that if it could do so, it is only by using another route of entry not considered here.

3.6 ASLV envelope pseudotyped HIV, N- and B-tropic MLV are not restricted by a range of Trim proteins after entering cells via Tva800

Trim5α is just one isoform of one member of a family of over 70 proteins containing all or parts of the tripartite motif and the B30.2 domain (Reymond et al. 2001), of which at least two other members have been confirmed to exhibit anti-viral activity. Trim1 has anti-N-MLV activity, and Trim19 (PML), found in nuclear bodies, is relocated under the influence of several viral infections, and is able to repress human foamy virus gene expression (Regad et al. 2001). Having confirmed that no novel anti-viral activity of Trim5α is evident when the virus entered via the two receptors, it was of interest to see if any other Trim proteins exhibited anti-viral activity if the virus took the more unusual post-entry route subsequent to binding to Tva800. Human Trim proteins 1, 6, 15, 18, 21, 22, 26, 27, 31, 34, 38, and 68 were cloned into pLgatewayIRESYFP by Dr. Melvyn Yap. $5x10^4$ d800 cells were plated and transduced at MOI ~1 with one of the human Trim proteins listed above. Three days later these were challenged with ASLV envelope-pseudotyped HIV, G89V HIV, NB-MLV, N-MLV and B-MLV. G89V HIV is mutated in the CA domain that binds cyclophilinA, so that it can no longer bind CA. This mutation has been noted to abolish restriction by Trim-Cyp, but is not absolutely required for Trim5α restriction (Stremlau *et al.* 2006b). After three days the percentage of virally infected d800 Trim-positive and d800 Trim-negative cells was assessed by FACS. The ratio of Trim-positive cells infected was divided by Trim-negative cells infected and again, a value of lower than 0.3 indicates significant restriction activity.

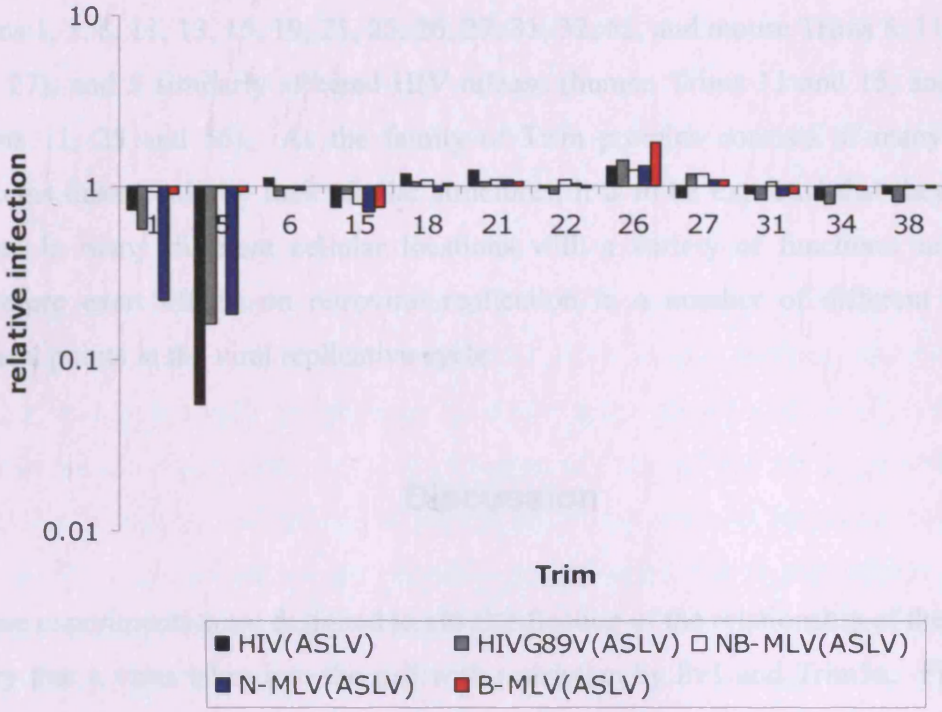


Figure 3.6 No new restriction is revealed when viruses encounter a panel of Trim proteins in d800 cells. d800 cells were plated and transduced with the trim proteins shown at MOI 1. 2 days later cells were split and challenged with ASLV Env pseudotyped HIV, HIV G89V, NB-MLV, N-MLV and B-MLV. Results shown are presented as the ratio of (proportion of trim positive cells infected)/(trim negative cells infected). A ratio of <0.3 reveals restriction activity.

As expected, expression of Trim5α led to a considerable drop in titres of HIV, N-MLV and G89V HIV. Titres of N-MLV were also reduced in cells expressing Trim1 as previously reported (Yap et al. 2004). For all other trim proteins used in this panel no effects on replication on any of the viruses could be detected. This would, of course, not completely preclude the possibility that anti-viral activity could manifest itself under different conditions, or when a combination of Trim proteins was expressed in the same cell.

An extensive screen has been recently carried out in which 55 Trim proteins were assessed for their abilities to alter ASLV Env pseudotyped HIV and MLV viral entry through the Tva950 receptor, as well as viral release (Uchil *et al.* 2008). No Trim proteins surveyed were found to have significant effects (>10-fold inhibition) against HIV or N-MLV entry, with the exception of human Trims 1 and 5 against N-MLV, confirming the results reported here. When release of virions was investigated,

however, 19 different Trim proteins inhibited MLV release by at least 10-fold (human Trims 1, 5, 8, 11, 13, 15, 19, 21, 25, 26, 27, 31, 32, 62, and mouse Trims 8, 11, 19, 25, and 27), and 5 similarly affected HIV release (human Trims 11 and 15, and mouse Trims 11, 25 and 56). As the family of Trim proteins consists of many diverse proteins linked only by their similar structures, it is to be expected that they will be found in many different cellular locations with a variety of functions, and could therefore exert effects on retroviral replication in a number of different ways at several points in the viral replicative cycle.

Discussion

These experiments were designed to aid clarification of the relationship of the route of entry that a virus takes into the cell with restriction by Fv1 and Trim5α. Firstly the receptors Tva800 and Tva950 were expressed in *Mus dunni* cells. The effects of NH_4Cl on ASLV Env-mediated entry into these cells was investigated, revealing a significant difference in the effects on entry via Tva800 and Tva950.

Mus dunni cells engineered to express Tva800 or Tva950 are fully permissive to infection with ASLV Env pseudotyped MLV cores. Entry via either receptor permits successful infection. However, when the infection is blocked using NH_4Cl, differences in the pathways used subsequent to receptor binding emerge. Viruses that have bound to Tva800 are stable for up to 6 hours under an NH_4Cl block and can still initiate a productive infection, whereas those that bound to Tva950, like virions bound to VSV-G, lose infectivity after 2 hours. There is no difference in the envelope protein used between Tva800 and Tva950 and therefore no difference between the fusion peptides, which is what anchors the envelope protein and virus into the target cell membrane. Therefore the difference must be due to the method of attachment of the receptor to the cell membrane, or the localised milieu of the plasma membrane in which the receptor resides and the way in which this affects the manner and rate of internalisation. This latter possibility is explored and discussed further in chapter 4.

GFP-labelled virions co-localise with cd63-positive compartments under an NH_4Cl-induced block, however it has not been possible to confirm the exact identity of this

intracellular compartment. Virions with a red fluorescent membrane label and GFP-vpr labelled nucleic acid can be used to detect when membrane fusion has occurred (with loss of the red label), and thus when a productive infection is likely to occur (used in experiments described in chapter 5). Usage of these dually labelled virions would help to guard against following virions through intracellular pathways that had been non-specifically endocytosed and were destined for lysosomal destruction. By infecting cells with these virions under an NH_4Cl block, and then fixing the cells and staining subcellular compartments with EEA1, cd63 or other markers, and then a third colour (blue), it might be possible to detect more specifically which subcellular compartment virions were in. A comparison of cells which had a constant NH_4Cl block up to fixation, and cells in which this block was removed for a short time before fixation to allow fusion, would hopefully reveal virion fusion and release near one type of compartment. Such experiments using a secondary antibody with emitted light wavelength 350nm, as this was the only third colour that did not carry over into different channels and could be satisfactorily resolved by the filterset on the deltavision microscope, were attempted. However, these experiments could not be pursued as the staining pattern of early and late endosomes was too indistinct to be able to draw any definite conclusions.

Ammonium chloride may have effects on the morphology of intracellular compartments and on trafficking routes that are as yet undefined. Another chemical that inhibits the acidification of endosomes is bafilomycin A1, which acts by blocking proton ATPases, but it has also been shown to block transport from early to late endosomes (Bayer et al. 1998). Thus inhibition of a virus by bafilomycin A1 could be due to the prevention of viral uncoating, or in an entirely separate manner to the inability of the virus to continue down the endosomal pathway and access the late endosome. Considering the results presented here and the ASLV-A Env-Tva receptor system, however, it is unlikely that in ammonium chloride-treated cells the virus is being blocked by anything other than an inability to initiate fusion in the endosomes. ASLV-A Env has been shown to require low pH for the conformational change necessary for fusion, and so even if ammonium chloride does affect trafficking downstream of this point of fusion, in the context of these experiments this would be irrelevant as the virus would be blocked in the endosome (Mothes et al. 2000; Barnard et al. 2006).

Examination of other systems where one receptor has two modes of attachment to the cell membrane indicate that the difference in attachment is not necessarily trivial, and there are difference in cellular pathways after entry that can significantly affect the timing and success of the infection. Viruses may have evolved to exploit a niche in these pathways, or be able to make use of several. There is no indication, however, that there is any physiological relevance of the usage of Tva800 or Tva950.

It has not been possible to pinpoint all the commonalities and differences in entry pathways subsequent to Tva800 and Tva950 binding. Both pathways are downregulated by dominant negative Rab5, which is involved in clathrin-mediated endocytosis (van der Bliek 2005), suggesting that this pathway plays at least some role in uptake via Tva800 and Tva950. This is consistent with previous data that showed dominant negative forms of dynamin, which mediates entry via both clathrin-mediated endocytosis and via caveolae, inhibited entry mediated by ASLV-A by between 60-80% (Mothes et al. 2000). However, this would mitigate the similarity somewhat of Tva800 and Tva950 to CD4-DAF and CD4-TM, as CD4-DAF was not taken up by a clathrin-mediated pathway. No differences were seen in the infectivity rate between the two receptors when the cells expressed dominant negative forms of Rabs 7, 9 and 11, which modulate later stages of the cellular pathways. The lack of effect seen with Rab7DN is surprising, given that this is thought to modulate traffic from the early to late endosomes. This may indicate the presence and usage of other trafficking pathways, or the insufficient down-regulation of Rab7 to generate a significant effect. Rabs 9 and 11 moderate traffic from the late endosome to the trans-Golgi network, and recycling between the trans-Golgi network and the early endosome respectively. It would therefore not be expected that virions bound to Tva800 or Tva950 would use pathways controlled by these Rab proteins during a successful infection, and therefore dominant negative inhibitors of these pathways would have no effect. Microscopy data indicate that Tva800 and Tva950 both deliver virions to late endosomes/MVB. NH_4Cl inhibits the fusion event between the viral and cellular membranes, the stage just before entry into the cytosol common to both receptors after binding to ASLV-A Env, so it is to be expected that the differences between the Tva800 and Tva950 pathways would be before this common point of fusion.

The possibility of either Tva800 or Tva950 rerouting ASLV Env pseudotyped virions around a block to infection in restrictive cells was assessed. $Fv1^n$, $Fv1^b$ and Trim5α were expressed in d800 and d950 cells, which were then infected with N-, B- and NB-MLV. The pattern of restriction was identical between the two receptors, which indicates that restriction is occurring either on both pathways or after the two pathways have combined, so that neither pathway permits the virus to evade the restriction. Testing a panel of Trim proteins against ASLV Env pseudotyped virions also failed to reveal any novel restriction, although all combinations of Trims and intracellular conditions cannot be exhaustively tested to rule out all anti-viral activity altogether.

There remains much to be clarified concerning the early stages of retroviral replication, and how these stages affect restriction. These studies, however, suggest that it is unlikely that variation in the route of entry would permit a virus to evade restriction factors, at least those of the type currently known and exemplified by Fv1 and Trim5α. Fv1 is associated with the trans-Golgi network (TGN) (Yap and Stoye 2003). Previous experiments using viral envelopes that delivered the virus either by internalisation or by direct fusion at the plasma membrane (ecotropic envelope or VSV-G and amphotropic envelope) did not reveal any difference in restriction activity of Fv1 against N- and B-tropic MLV that was route of entry-dependent (Jolicoeur 1979; Bock *et al.* 2000; Yap and Stoye 2003). This would suggest that an interaction of the virus with the TGN is crucial to a successful infection and occurs no matter how the virus enters the cell. Thus a restriction factor that is found in, or associated with, a subcellular compartment that a virus is obligated to pass through will be impossible for the virus to circumvent. During the period of these studies it was confirmed that Trim5α is cytoplasmic, and found in structures known as cytoplasmic bodies, although the role of these bodies in restriction is debated (Xu *et al.* 2003; Song *et al.* 2005; Campbell *et al.* 2007a). As the virus must penetrate the cytoplasm in order to undergo essential steps in replication, it is conceptually difficult to understand how any differences in route of entry prior to this stage would confer resistance to, or allow the virus to otherwise avoid, the restrictive effects of Trim5α.

The obligation to penetrate the cytoplasm makes it likely that restriction factors in general are very difficult, if not impossble, for the virus to evade, and adds to their

potency as anti-virals. It is, however, possible that a type of anti-viral with different characteristics to currently known restriction factors may be described, and that this could have a route of entry component. This would probably require the anti-viral to be non-cytoplasmic, and reside in a cellular compartment that the virus could pass through or evade, with different outcomes of infection accordingly.

Certain questions arise from these studies about the importance of viral binding domain and mode of uptake in determining viral entry characteristics. Tva800 and Tva950 have identical virus binding domains, but different entry kinetics and the uptake route differs. Viruses entering via VSV-G are also taken up by endosomes, and infection is blocked by NH_4Cl, although there is no two-step fusion process, as for ASLV Env. Some similarities are seen between the receptor for VSV-G and Tva950. Both are single membrane-spanning proteins. The different restriction profiles shown in figure 3.5.2 for both Tva800 and Tva950 correspond well to published data on Fv1 and Trim5α restriction against viruses pseudotyped with VSV-G (Bock et al. 2000). When a timed infection is carried out under an NH_4Cl-induced block, however, differences emerge (figures 3.2.2 and 3.2.3). The decrease in infectivity seen with viral binding to VSV-G is even more dramatic than that seen for Tva950. With VSV-G, after 1 hour approximately 40% of virions remain infectious, 2 hours 15% and 3 hours 6%. For Tva950 the figures are after 2 hours 74% of virions are infectious, and 4 hours 19%. There is a much more dramatic difference seen when either Tva950 or VSV-G is compared with Tva800, where 60-80% of virions remain infectious for 8 hours, but the difference between Tva950 and VSV-G is probably still significant.

Under normal cellular conditions, after exposure to low pH in the endosome, a hydrophobic region of VSV-G is exposed that would interact with the target membrane (Durrer et al. 1995). The insertion of the ASLV Env fusion peptide, however, takes places at neutral pH (Barnard et al. 2006) and the shift to lower pH merely completes the fusion reaction. This means that under an NH_4Cl-induced block, the envelope proteins would not be found in equivalent conformations. ASLV Env would already have been activated, and the fusion peptide firmly inserted into the target endosomal membrane. Fusion would not be completed until the pH drops, but in the interim the ASLV Env complex is deeply embedded into the membrane. As

VSV-G does not expose its hydrophobic region apart from at low pH, under endosomal neutralisation by NH_4Cl the virus-VSV-G-receptor complex would still be in the same conformation as it was at the plasma membrane, on the exterior of the cell, with no direct attachment between the virus and the target membrane, only indirectly through the receptor. The small difference in the stabilities of virions bound to Tva950 and VSV-G could be largely due to this difference.

The extraordinary stability of virions bound to Tva800 then comes into question, as the receptor is clearly significantly different to Tva950 despite interacting with the same viral envelope protein, the conformation of which would obviously be the same under an NH_4Cl-induced block. The stability must relate to either the method of membrane attachment, or to the indirect effects of this attachment, namely the area of the plasma membrane where these receptors reside. GPI-anchored proteins (GPI-APs) cluster in lipid rafts, and Tva800 has been shown to localise to these rafts (Mothes et al. 2000). After caveolin-1-mediated endocytosis, GPI-APs have been suggested to pass through a variety of endosomal compartments, the exact nature of which probably depends on the specific cell type under investigation. It was shown that in BHK cells some GPI-APs localise to late endosomes, and that when purified these contained lipid rafts (Fivaz et al. 2002). This would suggest that the integrity of lipid rafts is to some extent maintained after endocytosis from the plasma membrane, while passing through intracellular compartments (Miaczynska and Zerial 2002). This lipid raft, therefore, would be the target membrane into which ASLV Env inserts the fusion peptide after binding to Tva800. The different characteristics of this type of membrane compared to non-raft areas will affect the durability of the interaction, and probably also the persistence of the virion under a block to infection. Crucially, it has been suggested that lipid rafts are excluded from entering degradative compartments in some cell types, which fits well with viruses bound to the lipid raft-associated Tva800 being less susceptible to degradation than Tva950 under the NH_4Cl block (Kobayashi et al. 1998; Mayor et al. 1998).

A diagrammatic conclusion is shown in fig 3.7. This is a schematic of the three pathways, and shows the three potential receptors for ASLV Env and VSV-G pseudotyped virions. Also shown are the routes taken after binding to one of these receptors, and what is known of the controlling or inhibitory factors for each route.

The intracellular compartments accessed by the different pathways are shown in different colours, although it should be noted that these could be different areas of the same compartment. For example, the Tva800 receptor could be found in a microdomain of a sub-cellular compartment, with Tva950 and the receptor for VSV-G outside these microdomains. When vesicles form from areas of this compartment for transport and degradation in the lysosome, the lipid raft microdomain and Tva800 would be excluded, Tva950 and the receptor for VSV-G included. Thus despite accessing the same intracellular compartment, their ultimate fate would be different.

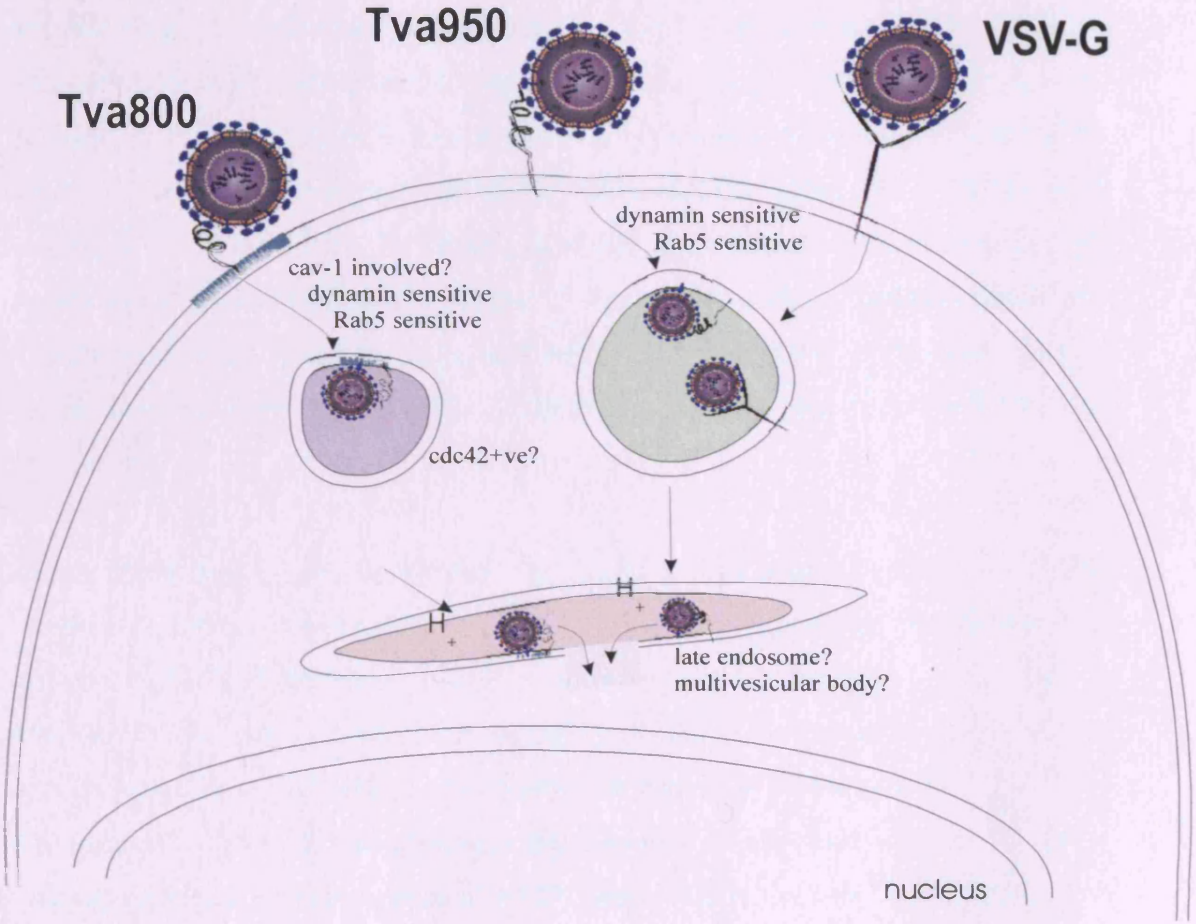


Fig. 3.7 Schematic representation of three possible entry pathways via VSV-G and receptor, ASLV Env and Tva800, or ASLV Env and Tva950. The little that is known about each route is shown, but cellular compartments remain to be clarified. Each compartment shown in a different colour represents a potentially separate identity and constitution. References for each pathway are as follows: Tva800 (Keller *et al.* 1992; Mothes *et al.* 2000; Sabharanjak *et al.* 2002; Narayan *et al.* 2003), Tva950 (Narayan *et al.* 2003), VSV-G (Aiken 1997; Sun *et al.* 2005)

The difference between Tva800 and Tva950 may be explained by the different type of membrane in which the receptors reside which have different biophysical properties,

and different intracellular fates after sorting. The difference between Tva950 and the receptor for VSV-G, despite the two receptors residing in the same area of membrane, is due largely to the conformational state in which the envelope protein that binds to these receptors is in when acidifcation of the endosome is prevented.

Chapter 4

ASLV-A Must Bind More Than One Tva800 for Entry

As discussed in the introduction and chapter 3, Tva800 is the GPI-anchored form of the receptor that mediates entry of ASLV-A into cells. Fusion mediated by ASLV-A Env occurs via a unique two-step process, in which binding of envelope to the receptor triggers conformational changes that expose the fusion peptide, which is inserted into the cell membrane. Completion of fusion requires a drop in pH, such as is found in the acidic environment of endosomes (Mothes et al. 2000; Barnard et al. 2006). Tva950 is a receptor that shares 100% identity with Tva800 in the viral binding domain, but differs in the way in which the receptor is attached to the cell membrane at its C-terminus. In chapter 3 it was shown that although Tva800 and Tva950 bind ASLV Env identically, and exactly the same fusion mechanism follows, the pathway by which the virus then enters the cell differs, due to this difference in attachment.

During these studies, routine cloning was carried out in order to insert the Tva800 receptor into the vector pLgatewayIRESYFP, creating pLgatewayTva800IRESYFP (called Tva800-YFP hereafter). In order to assess whether expression of Tva800 in *Mus dunni* cells was sufficient to permit entry of ASLV Env pseudotyped viruses, a panel of *Mus dunni* cell lines were created that had been transduced with clones of this plasmid. One cell line generated from a clone of the plasmid (clone 1) gave strikingly different titration curves of ASLV Env pseudotyped NB-MLV compared to the other clones. As this difference was replicable, the clone was investigated further and compared to one of the other clones (clone 4), which generated a more generic titration curve. The results are discussed here in relation to a possible model for ASLV entry.

Results

4.1 Entry of ASLV pseudotyped virus via two clones of Tva800 receptor vector gives two different titration curves

In order to ensure that *Mus dunni* cells could express Tva800 and that it was able to function as a receptor for ASLV Env pseudotyped viruses, the vector Tva800-YFP was made to allow transient tests for receptor function. This was performed before the construction of the d800 cell line described in chapter 3. Successful transduction of the cell, and by extension receptor production, can be detected by assay for YFP expression. Several clones were tested, and one clone in particular (clone 1) gave a strikingly different titration curve when increasing volumes of ASLV-Env/NB-MLV were added to cells, as shown in figure 4.1.

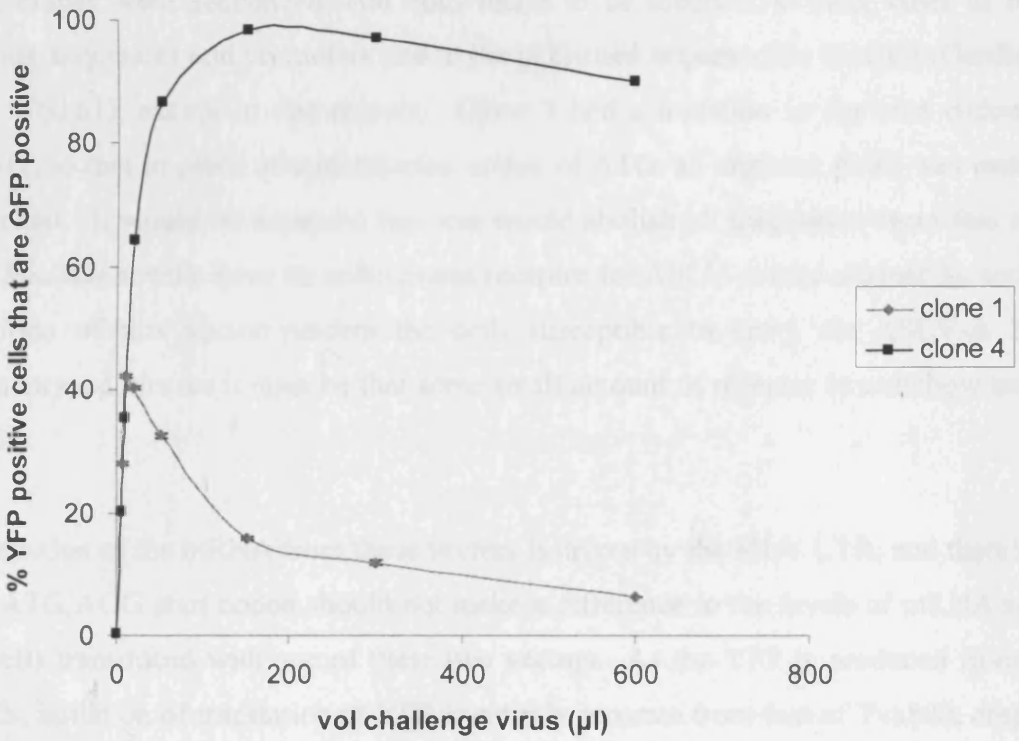


Fig. 4.1 Increasing titres of ASLV Env pseudotyped NB-MLV on cells expressing clone 1 gives an anomalous titration curve. *Mus dunni* cells were plated at $5x10^4$ cells per well, and transduced with Tva800-YFP clone 1 or Tva800-YFP clone 4 vectors at MOI 1. Two days later cells were passaged, and after one further day were challenged with increasing volumes of ASLV-Env/NB-MLV. The percentage of cells successfully infected as a function of YFP positive cells is shown.

It would normally be expected that increasing the volume of virus applied to the cells would increase the proportion of cells becoming infected. At low volumes this appeared to be the case, and the increase from 2-10µl appeared to correspondingly increase the proportion of virus infecting cells for both clones. Above this volume, the graphs diverge. At titres above 20µl for cells transduced with Tva800-YFP clone 1, the percentage of cells that were successfully infected began to fall, until at volumes over 150µl less than 15% of cells were successfully infected. However, for cells transduced with clone 4 increasing titres continued to increase the percentage of cells infected to over 95% with 200µl. As the odd curve with clone 1 was obtained in two independent experiments carried out with different batches of vector, it was investigated further.

The clones were sequenced, and both found to be identical to each other in both coding sequences and promoters and to the published sequence for Tva800 (GenBank ID: 403161), except in one respect. Clone 1 had a mutation in the start codon of T→G, so that in place of a methionine codon of ATG, an arginine AGG was instead encoded. It would be expected that this would abolish all translation from this site, yet *Mus dunni* cells have no endogenous receptor for ASLV-A (see chapter 3), and as addition of this vector renders the cells susceptible to entry via ASLV-A Env pseudotyped viruses it must be that some small amount of receptor is somehow being made.

Production of the mRNA from these vectors is driven by the MLV LTR, and therefore the ATG/AGG start codon should not make a difference to the levels of mRNA seen in cells transduced with one of these two vectors. As the YFP is produced from an IRES, initiation of translation of YFP is entirely separate from that of Tva800, despite being from the same mRNA. It would, therefore, be expected that the number of cells YFP-positive after infection with the same volumes of Tva800ATG and Tva800AGG would be roughly equivalent, and this is what was found (not shown). However, initiation of translation of Tva800 would differ, as this is the stage at which the AGG mutation would manifest itself, and affect protein production. It is difficult to understand conceptually how any protein is produced at all, given that non-canonical start codons are rare, yet as virions are patently able to enter cells transduced with

Tva800AGG the evidence remains that some protein must be produced. It could then be asked whether the (unknown) consequences of this codon mutation are responsible for the odd shape of the titration curve seen.

In the paper describing the characterisation of the quail homologue of *Tva* it was reported that during cloning the promoter was accidentally deleted, but that even with the resultant inefficient expression of Tva the cells were still susceptible to infection mediated by ASLV Env (Bates et al. 1993). Over-expression of the receptor in naturally permissive avian cells rendered them 100x more sensitive. Thus, there is a precedent for small amounts of receptor being sufficient for a baseline level of viral entry. However, this does not explain why increasing viral titre at first increases, but then reduces the actual level of virus getting into the cells.

There are previously described atypical viral titration curve phenomena in the literature. One example occurs during antibody dependent enhancement (ADE) of infection of dengue virus, in which addition of increasing antibody causes at first an increase, and then a subsequent decrease in infection levels of a fixed titre of virus on Fc-receptor bearing cells *in vitro* (Halstead 1982; Morens et al. 1987; Goncalvez et al. 2007). This is because the antibody surrounds the viral particle initially enhances the interaction with Fc-receptors on the target cells, or induces uptake by endocytosis. As the level of antibody increases beyond this, however, the interaction of the dengue virus with the target cell is increasingly blocked and titres start to fall rapidly.

In another example, when amphotropic MLV particles are titrated onto cells, increasing the number of MLV envelope proteins per virion increases the number of cells successfully infected up to a point, after which a decrease in infected cells is seen as envelope numbers further increase (Landazuri and Doux 2007). When low titres were used, the latter decrease was not seen, solely a plateau in the number of cells infected (Bachrach et al. 2000). The high doses of virus likely contain a large amount of inhibitory soluble Env, which would be diluted out in the end point titration assays used in the second study. However, studies in which viral stocks were purified of soluble factors (designed to mimic conditions of gene therapy protocols) also reproduced these results, indicating that virus-associated membrane proteins are

capable of blocking infection of neighbouring virions by steric hindrance, as well as soluble Env proteins (Landazuri and Doux 2007).

However, these examples are different from the phenomenon described here, as in both examples the level of virus stays the same, but other factors change concentration e.g. antibody, envelope number per virion. In experiments described here, the variable is the actual volume of virus placed on the cell, so the differences seen in the number of cells infected must directly relate to the number of virions potentially able to infect the cell or some other factor in the medium that influences infection.

4.2 Increasing the level of eyp800 vector increases the proportion of viral entry

If the assumption is made that the AGG mutation results in a very limited level of Tva800 at the cell surface, it can be hypothesised that this is a limiting factor causing the rate-dependent step of viral entry. In this case, an excess of virus at the cell surface (as occurs when the volume is >10µl) results in a block to entry. An excess of virus should result in up to 100% receptor occupancy, and the lack of unoccupied receptor would prevent further viral entry. It is then logical to suggest that if virus binding of one receptor is insufficient for successful viral entry (c.f. less than 15% cells transduced at titres over 150µl) then multiple receptor binding events by one virus are necessary for entry, presumably mediated by multiple Env trimers on the virus.

A very simple way to test this hypothesis would be to increase the level of Tva800 in the cells to see if the titration curve approaches 100% infected cells at higher levels of Tva800 and challenge virus. It would be assumed that this is actually the case for clone 4 (the clone with the canonical ATG start codon), but it should be possible to show this with clone 1. Four populations of cells were transduced with clone 1 (Tva800AGG) vector at MOI of 1, 5, 10 or 20, and then challenged with ASLV Env pseudotyped NB-MLV. The resulting titration curves are shown in fig 4.2

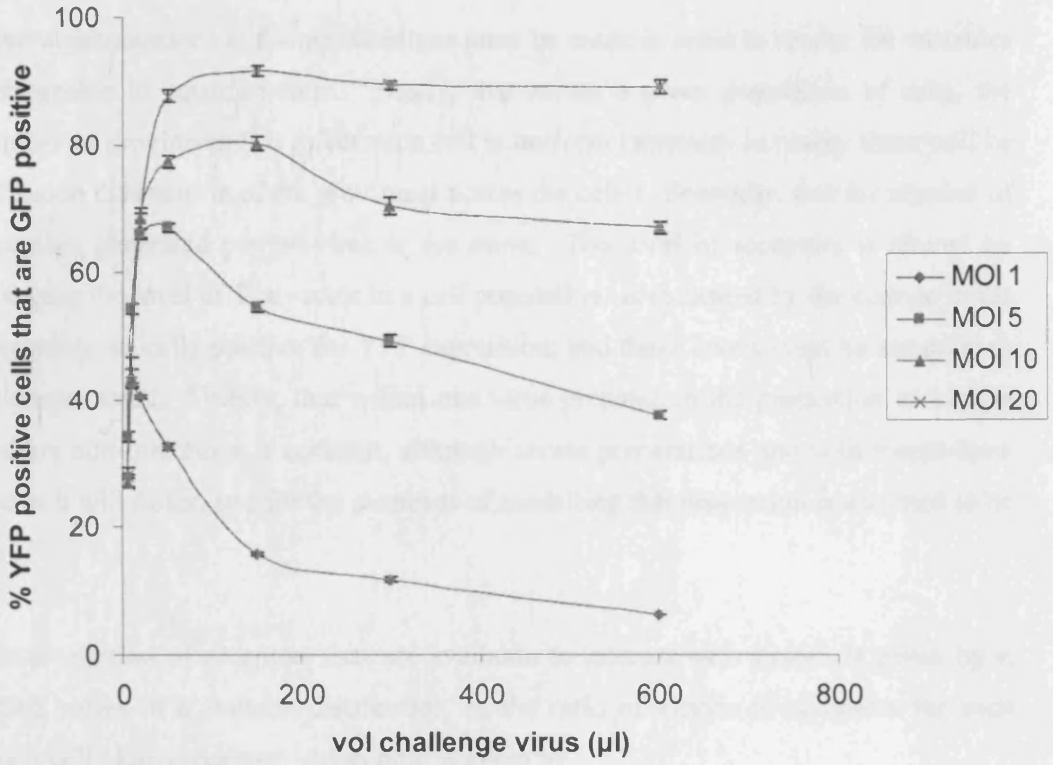


Fig. 4.2 Increasing the MOI of Tva800-YFP vector in cells increases the number of ASLV-Env/NB-MLV virions that are able to enter. *Mus dunni* cells were plated and transduced with Tva800-YFP AGG vector (i.e. clone 1) at different MOI as shown. Cells were split and challenged after 3 days with ASLV-Env/NB-MLV. Results shown are combined from two separate experiments, and error bars represent sdm.

From these data it can be clearly seen that as the level of receptor in the cell increases, the permissivity of that cell to high titres of viruses increases, which supports the assumption that low receptor level is the limiting factor of viral entry at high virus levels.

4.3 Viral entry dependent on receptor availability can be modelled using a Poisson distribution

Given the above experimental data, it should be possible to model the percentage of virus entering cells mathematically as a function of the level of Tva800 at the cell surface, and the total volume of virus. The modelling detailed below was done by Professor John M Coffin, Tufts University, USA.

Several assumptions and simplifications must be made in order to render the variables manageable in equation form. Firstly, that across a given population of cells, the number of proviruses that infect each cell is uniform (although in reality there will be a Poisson distribution of the proviruses across the cells). Secondly, that the number of receptors generated per provirus is the same. The level of receptors is altered by changing the level of Tva vector in a cell population, as indicated by the change in the percentage of cells positive for YFP expression, and these levels must be assumed to be proportional. Thirdly, that within one virus preparation the proportion of virions that are non-infectious is constant, although across preparations and with freeze-thaw cycles it will differ, and for the purposes of modelling this proportion is assumed to be zero.

The proportion of receptors that are available to interact with virions is given by r, which varies in a Poisson distribution on the ratio of virions to receptors for each target cell. Each receptor: virion ratio is given by

$$\frac{\text{(relative no. receptors per cell x no. receptors per MOI)}}{\text{(titre of challenge virus x no. virions per µl).}}$$

The number of cells that are successfully infected is related to the number of virions that can interact with the required number of receptors on those target cells, which in these models is taken to be between 1 and 4. Thus, this number of target cells which have a virion interacting with the required number of receptors is given by $1-\Sigma$ (Poisson terms $0...r-1$ for each receptor: virion ratio), and the proportion of cells infected is given by $1-e^{(\text{MOI GFP}^{+}\text{proportion of cells with available receptors})}$ (Please see appendix 1 for a fuller explanation of these equations and workings, with symbols).

Using the above equations a theoretical experiment can be carried out in which the level of Tva800 is varied between cell populations, and different titration curves obtained. The results are shown in figure 4.3.

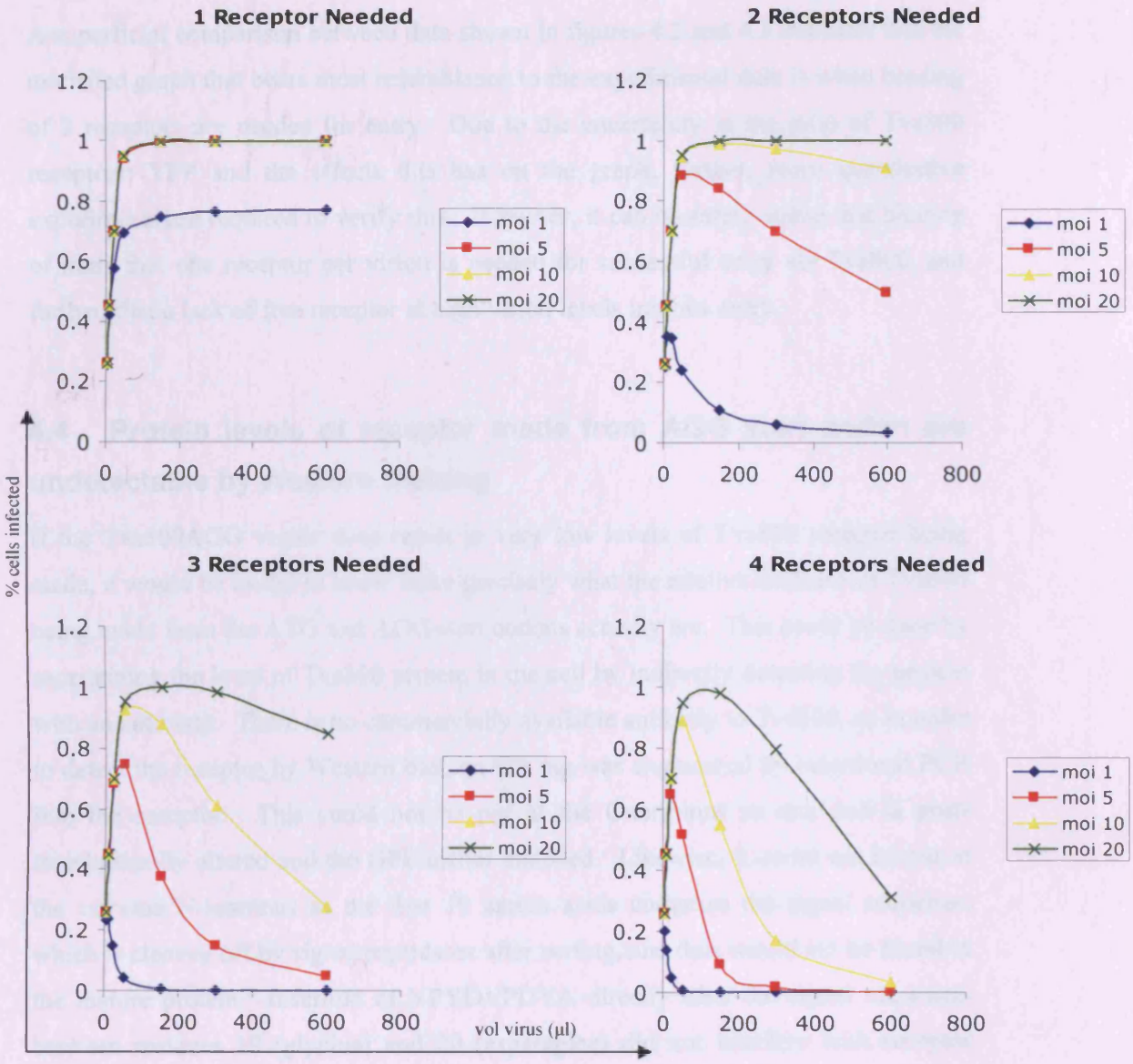


Fig. 4.3 Modelling using a Poisson distribution of the relative levels of cells infected (Y-axis) when titrating virus (µl, X-axis) onto cells expressing different levels of receptor. The top-left graph assumes that binding of 1 Tva800 receptor is necessary for a virus to enter a cell, the top right that 2 receptors are needed, and the bottom left and right 3 and 4 receptors respectively.

There is one major assumption used in the calculations, which is the actual number of Tva800 receptors for every MOI YFP, assumed to be 1.5 in the graphs shown above. This could potentially vary between experiments but it is difficult to know exactly what it is, aside from that is must be >0, as otherwise no receptor would be produced at all, but it is difficult to see how it could be >>1 due to the AGG mutation. An illustration of how this factor changes the shape of the curves obtained is shown in appendix 2.

A superficial comparison between data shown in figures 4.2 and 4.3 indicates that the modelled graph that bears most resemblance to the experimental data is when binding of 2 receptors are needed for entry. Due to the uncertainty in the ratio of Tva800 receptors: YFP and the effects this has on the graph, further, more quantitative experiments are required to verify this. However, it can be safely stated that binding of more that one receptor per virion is needed for successful entry via Tva800, and further, that a lack of free receptor at high virion levels inhibits entry.

4.4 Protein levels of receptor made from AGG start codon are undetectable by Western blotting

If the Tva800AGG vector does result in very low levels of Tva800 receptor being made, it would be useful to know more precisely what the relative amounts of Tva800 being made from the ATG and AGG start codons actually are. This could be done by ascertaining the level of Tva800 protein in the cell by indirectly detecting the protein with an antibody. There is no commercially available antibody to Tva800, so in order to detect the receptor by Western blot, an HA tag was engineered by insertional PCR into the receptor. This could not be put at the C-terminus as this end is post-translationally altered and the GPI anchor attached. Likewise, it could not be put at the extreme N-terminus as the first 19 amino acids comprise the signal sequence, which is cleaved off by signal peptidases after sorting, and thus would not be found in the mature protein. Insertion of YPYDVPDYA directly after the signal sequence, between residues 19 (glycine) and 20 (asparagine) did not interfere with receptor function and by extension, receptor production and conformation. The new sequence was run through SignalP 3.0, which detects signal peptide cleavage sites (Bendtsen *et al.* 2004), and the output was identical to the original sequence indicating that the HA tag should not interfere with signal peptide processing. Similarly, when the start codon of the HA-Tva800 sequence was AGG, cells transduced with this vector produced the same a-typical titration curve as Tva800AGG (figure 4.4.1), and so was deemed suitable for use in detection of receptor levels. HA-Tva950 ATG and HA-Tva950 AGG were also constructed.

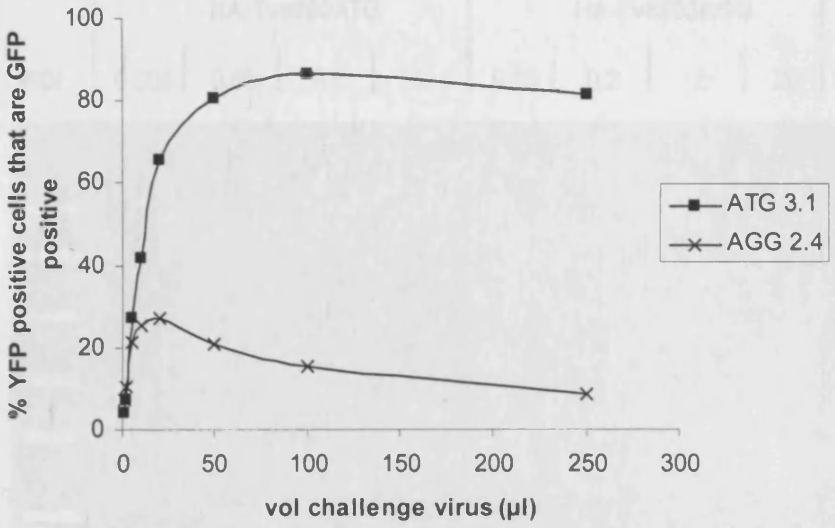


Fig. 4.4.1 Use of the HA-Tva800 vectors replicate the titration curves generated from Tva800ATG and Tva800AGG. *Mus dunni* cells were transduced with the HA-Tva800 vectors, then challenged 3 days later with ASLV Env pseudotyped NB-MLV. The legend shows whether the start codon of the Tva800 vector was ATG or AGG, and the MOI at which the cells were transduced. Results shown are representative of three independent experiments, as variations in MOI obtained each time made them difficult to combine.

Mus dunni cells were transduced with HA-Tva800ATG and HA-Tva800AGG at different MOI and harvested after 3 days. Cells were lysed, and assayed by Western blot for protein expression. The cells transduced with HA-Tva800ATG had final MOI of between 0.006-6, and those transduced with HA-Tva800AGG of between 0.02-20, both assayed by YFP expression. Samples were probed with anti-HA and GFP antibodies, then stripped and normalised by anti-GAPDH. A sample of untransduced *Mus dunni* cells was included as a control.

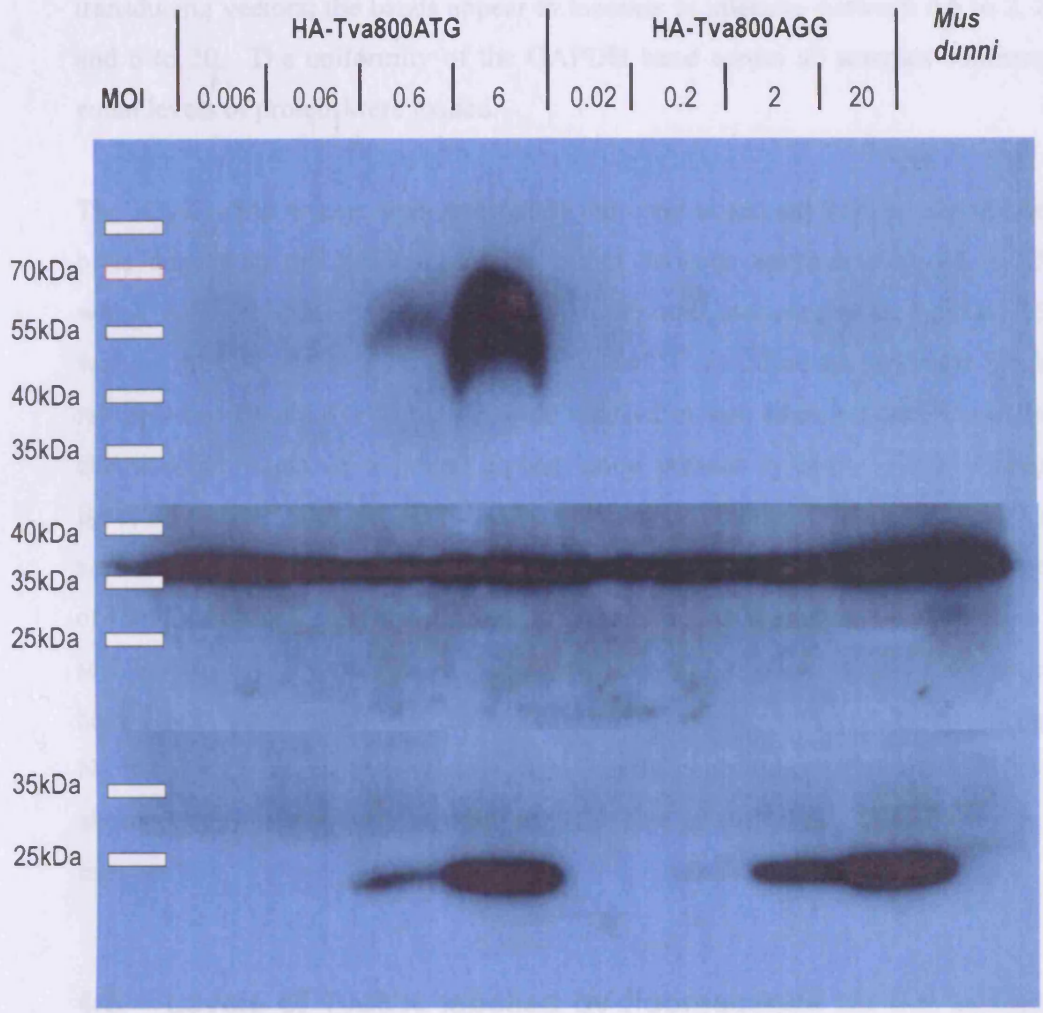


Fig. 4.4.2 Western blot of *Mus dunni* cells transduced at different MOI with HA-Tva800 ATG and HA-Tva800 AGG. *Mus dunni* cells were seeded in 60mm dishes and transduced at the MOI shown with one of the two vectors. Cells were grown to confluence, then lysed with 1xSDS loading buffer, denatured, and separated on a gel as shown above. The blot was probed with anti-HA (top row), anti-GAPDH (middle row, loading control) and anti-GFP (bottom row). Markers shown MW.

No HA or YFP expression was detected in lanes that were applied with *Mus dunni* cell lysates, which were used as a negative control. Of the transduced samples, only those cells transduced with HA-Tva800ATG were detectable by Western blot for HA expression (i.e. for receptor expression) and only when the MOI was at least 0.6. Cells transduced with HA-Tva800AGG did not produce detectable levels of receptor, even when the MOI was ~20. The intensity of the bands produced by probing for YFP expression are of the relative intensities that would be expected from the MOIs of the

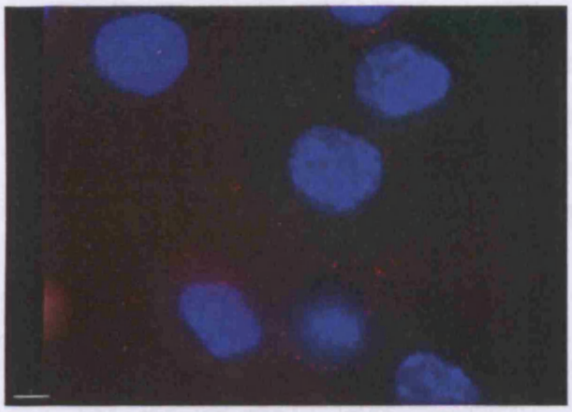
transducing vectors; the bands appear to increase in intensity between 0.6 to 2, 2 to 6 and 6 to 20. The uniformity of the GAPDH band across all samples confirms that equal levels of protein were loaded.

The HA-Tva800 protein was detected in the band at around 55kDa. In all Western blots done with this protein the size varied between approximately 45 to 55kDa, which is larger than expected from the amino acid sequence alone (around 15kDa, without the GPI anchor). Previous blots of Tva950 identified the protein in bands that ran between 29 and 43kDa, which were reduced in size when produced in cells with chemical inhibitors of N-linked glycosylation present by about 10kDa (Rong and Bates 1995; Balliet et al. 1999). Glycosylation prediction software predicts 3 and 6 sites of N- and O-linked glycosylation, respectively (Bendtsen et al. 2004). Digestion of HA-Tva800 cell lysates with N-glycosidase F reduced the size of the protein band seen by between 8 and 13kDa, but digestion with O-glycosidase did not appear to have any effect (not shown). This is consistent with previous results suggesting that N- but not O-linked glycosylation significantly contributes to the size of Tva800, although it does not fully explain why the size is still about 8-10kDa larger than expected.

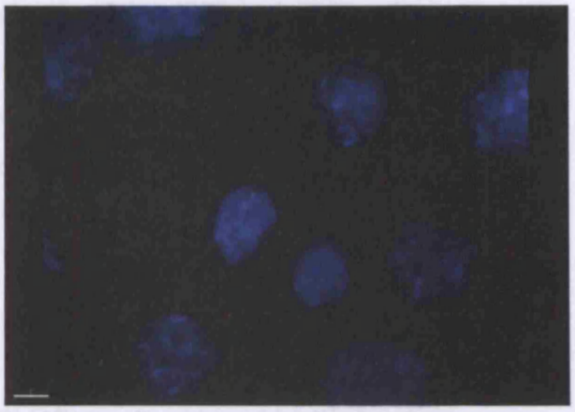
4.5 Levels of Tva800 labelled by fluorescence on the surface of cells are visibly lower

Probing by Western blot permits a comparison of total protein content between lysates from different cells. As Tva800 is a cell-surface receptor, it was of interest to see if there was a visible difference between levels at the cell surface, and if the difference in total Tva800 protein synthesis between cells transduced with Tva800ATG and Tva800AGG also translated to the cell surface levels. In order to do this, an hybrid protein comprised of the SU of ASLV Env linked to rabbit immunoglobulin G (SUA-rIgG) was used as a primary antibody. This was developed to assess the influence of different mutations in Tva on binding coefficients (Zingler and Young 1996). SUA-rIgG binds to Tva receptors but lacks the fusion peptide so will not initiate any further events typical of ASLV Env. *Mus dunni*, d800 or d950 cells were plated sparsely on coverslips and allowed to attach overnight before staining at 4°C the following day. SUA-rIgG was bound and then a fluorescent secondary antibody (Alexa Fluor 594)

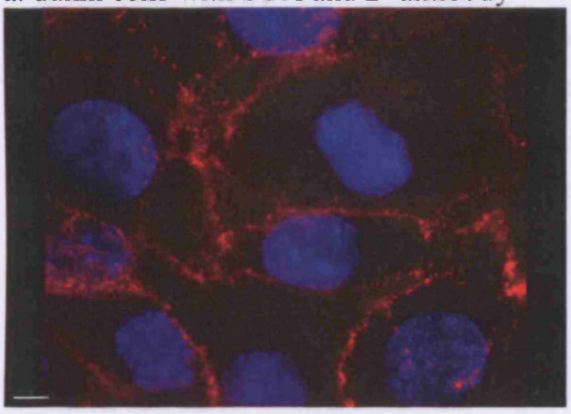
raised against rabbit IgG was bound. Cells were inspected under the microscope to assess the different levels of red staining seen on the cell surface. The immunoadhesin was generously provided by James Bruce.



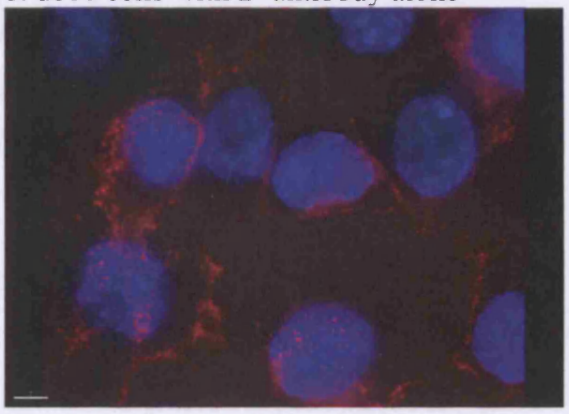
a. dunni cells with SUA and 2' antibody



b. d800 cells with 2' antibody alone



c. d800

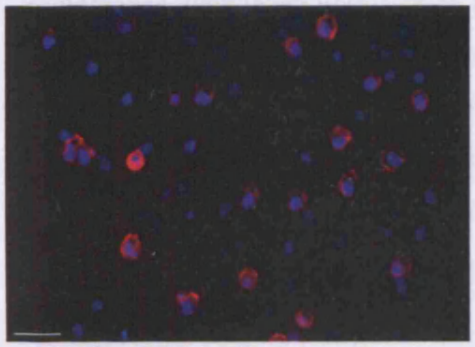


d. d950

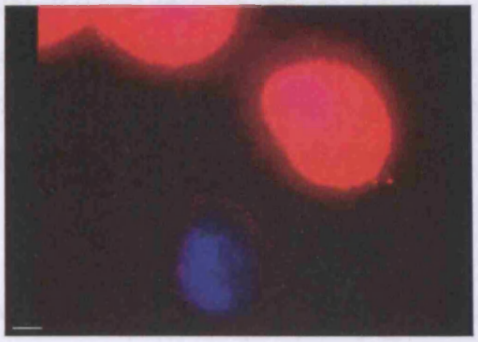
Figure 4.5.1 Specific staining of the Tva800 and Tva950 receptors can be achieved using the immunoadhesin SUA-rIgG and an anti-rabbit secondary antibody. Cells were plated at about 20% confluency onto glass coverslips, and allowed to settle overnight. They were washed, blocked at 4°C with 1% BSA in PBS, then incubated at 4°C in medium containing excess SUA-rIgG, washed again 3x with 1% BSA in PBS, then incubated with 0.8µg Alexa Fluor 594 in 0.5ml 1% BSA in PBS at 4°C for 40 minutes. Cells were then fixed, permeabilised and stained with DAPI. Shown are a) dunni cells stained with both SUA-rIgG and secondary antibody (negative control), b) d800 cells stained with secondary antibody only, c) and d) d800 and d950 cells, respectively, both stained with SUA-rIgG and secondary antibody.

The pictures in figure 4.5.1 show the control slides prepared to assess background staining and positive controls. The dunni cells stained with SUA-rIgG and secondary antibody and the d800 cells stained with secondary only provide proof of specific binding both of the SUA-rIgG, and of the secondary antibody to the SUA-rIgG. The contrast between these two pictures and the staining of the d800 and d950 cells is clear; in the latter pictures there is punctate red staining around every single cell in a radius that is approximately where the plasma membrane of the cells would be expected. Every cell should be stained as these cells are single cell clones, and the plasmid maintained by selection in G418. These, then, are the positive controls for staining of Tva receptors by SUA-rIgG.

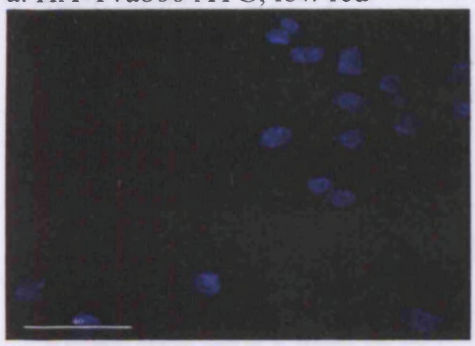
This staining was then applied to try to see how much receptor was produced in cells expressing the Tva receptors from the mutant AGG start codon. *Mus dunni* cells transduced with HA-Tva800 ATG, HA-Tva800AGG, HA-Tva950 ATG and HA-Tva950 AGG were prepared. Cells were transduced with HA-Tva800 ATG at MOI of <1, HA-Tva800 AGG at MOI of >20, HA-Tva950 ATG at MOI <1 and HA-Tva950 AGG at MOI >20. Once plated cells were stained as described for figure 4.5.1.



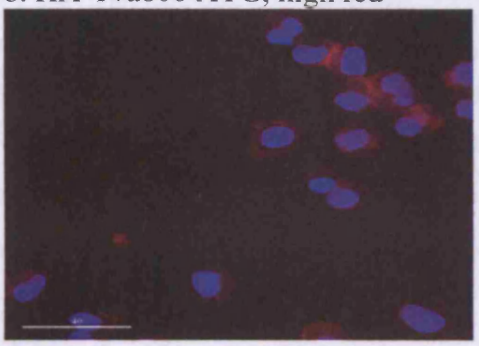
a. HA-Tva800 ATG, low red



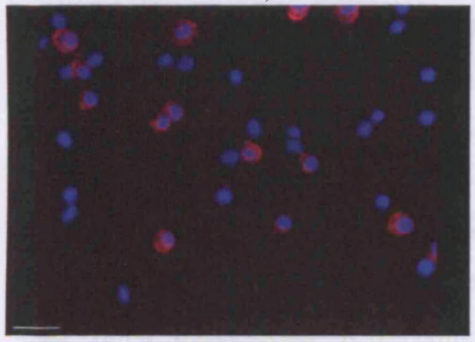
b. HA-Tva800 ATG, high red



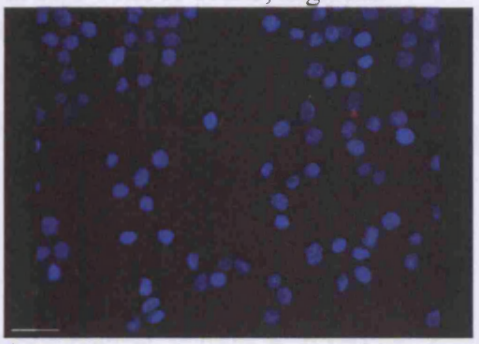
c. HA-Tva800 AGG, low red



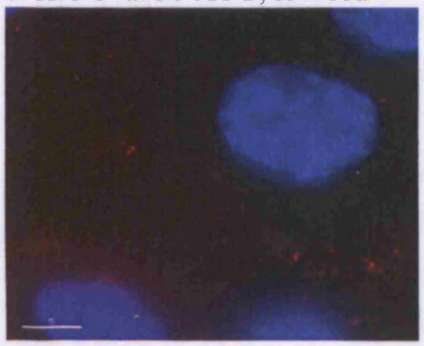
d. HA-Tva800 AGG, high red



e. HA-Tva950 ATG, low red



f. HA-Tva950 AGG, low red



g. dunni, high red

Fig. 4.5.2 Dunni cells transduced with Tva800 ATG, Tva800 AGG, Tva950 ATG and Tva950 AGG reveal different levels of red fluorescence correlating to different levels of Tva receptor at the cell surface. Cells were prepared as described for figure 4.5.1. During analysis of the red signal, the picture was either left un-enhanced or artificially brightened for the 'low' and 'high' descriptors respectively, to highlight low levels of fluorescence. Shown are *Mus dunni* cells transduced with: a) HA-Tva800 ATG, low red; b) HA-Tva800 ATG, high red; c) HA-Tva800 AGG, low red; d) the same frame as c, high red; e) HA-Tva950 ATG, low red; f) HA-Tva950 AGG, low red. A picture of *Mus dunni* cells stained with SUA-rIgG and secondary antibody, with a signal enhanced to similar levels to those in b and d is shown for comparison in g. Size bars are 5μM (b and g) or 40 μM (a, c, d, e and f).

Photographs were taken with a fixed time of 0.562 secs for the red channel, but then visualised either at 'low red' (un-enhanced), or 'high red', where the red channel was enhanced to try to make low-level staining artificially visible. This allows the contrast between cells expressing high and low levels of the receptors to be highlighted.

The first notable point is that as the cells transduced with the ATG plasmids (HA-Tva800 ATG and HA-Tva950 ATG) were infected at MOI <1, this is equivalent to up to 40% of the cells becoming infected. Therefore about 40% of the cells in the pictures a) and e) are capable of expressing Tva800 or Tva950. Red fluorescence, indicating easily detectable levels of Tva800 or Tva950, is seen in ⅓ of the cells in 4.5.2.a and e. This suggests that if a cell is infected by vector encoding either of the two receptors with an ATG start codon then the receptors are readily and efficiently detectable.

However, the cells transduced with the AGG plasmids were infected at MOI >20, which means that the percentage of cells able to express the Tva receptors is >95%. Cells infected with HA-Tva800 AGG, however, showed no sign of red staining when photographed under the 'low red' conditions, which brought up clearly visible staining for HA-Tva800 ATG. When the red signal was artificially raised ('high red'), staining became faintly discernible, as shown in picture 4.5.2.d. The background fluorescence of the slide also becomes visible, although the staining of the cells would appear to be somewhat distinguishable from this by the heightened fluorescent shapes around the cells. All cells appear to have this penumbra, which would be expected from the high MOI at which they were infected if this was indeed genuine staining of Tva receptors. However, it would also be expected from a low level of non-specific binding. Hence, it is difficult to know from this study alone how significant the shadowy halo of red staining actually is. Cells that are not expressing any Tva receptors exhibit only a non-specific level of staining (for example, the dunni cells stained in picture 4.5.1.a). When pictures of cells infected with HA-Tva800 ATG are adjusted to the 'high red' level, the signal from Tva800-expressing cells overwhelms the camera and appears as an overexposed red blob, for example as in 4.5.2.b. In that same picture is a cell that was probably not expressing Tva800 (given that <40% of the cells in this sample were positive). However, the heightened red

signal does appear to show a similar punctate pattern of staining similar to that seen for the HA-Tva800 AGG infected cells. This makes it very difficult to know how significant the faint staining in picture 4.5.2.d really is, and whether it is actually any different from background staining. Hence, while it is not possible to quantitate the difference in levels from these pictures, it is clear that it is significant. A similar case can be made for the cells infected with HA-Tva950 ATG and AGG plasmids, shown in the pictures e and f in figure 4.5.2.

4.6 Viral binding and entry can be blocked

In the experiments described in sections above, viral doses comprised solely of virions carrying GFP vector, which were competing with each other for receptor. Presumably, these doses also carried a proportion of non-infectious vector but this is very difficult to calculate for, as it will not be the same between batches of virus and experimental conditions. In order to try and quantitate the numbers of receptors at the cell surface and also the numbers of virions able to enter, an experiment was carried out in which a fixed volume of virions carrying GFP vector was titrated onto cells in competition with virions carrying no vector, so called 'empty virions'. These empty virions will be able to bind to, and enter cells, but as they carry no GFP vector the infection will not be recorded. Thus the empty virions can compete with GFP virions for receptors. Increasing the level of empty virions compared to a fixed volume of GFP virions should result in increasing competition for free receptor, and hence a decrease in GFP fluorescence. Ideally this would be done with three colours; YFP to monitor Tva800 levels, and a comparison between the fixed volume of GFP virions and an increasing volume of virions of a third colour made, but this is not currently possible. Cells were challenged with mixes of different proportions of GFP and empty virions made up to a fixed volume in PBS, and the percentage of GFP virions entering successfully was assayed.

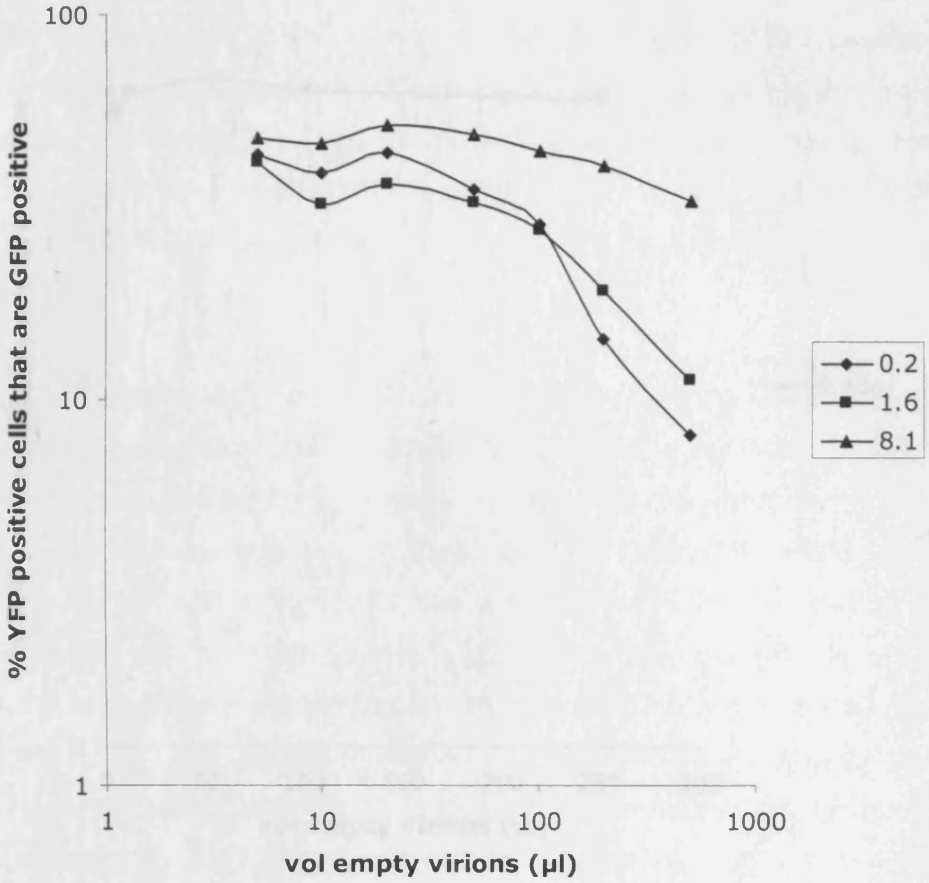


Fig. 4.6.1 Empty virions can successfully compete with virions carrying GFP vector, and can block infection. As the empty vector will still initiate entry into cells, receptors are only temporarily blocked. *Mus dunni* cells were transduced with Tva800 AGG vector at MOI 0.2, 1.6 and 8.1, and infected with 510µl fluid containing 10µl GFP virus mixed with between 0-500µl empty virions. Shown is the proportion of cells that became GFP positive as a function of YFP positive cells. The experiment was repeated twice but due to differences in YFP fluorescence levels obtained each time, the data from one experiment are shown.

The data in figure 4.6.1 show that titres of virus carrying GFP vector can indeed be successfully reduced by competition with empty virus. Titres were an initial level of 45-50% on cells expressing YFP, at all MOIs of Tva800 transduction. When there was a 50x proportion of empty virus compared to GFP virus (i.e. when the volume of empty virus was 500µl), titres were then reduced to <10% for the lowest levels of Tva800 AGG, (MOI 0.2), to 11% for MOI 1.6, and 32% for MOI 8.1. This indicates that the more Tva800 there is on the cell surface, the less susceptible that cell is to competition from empty virus.

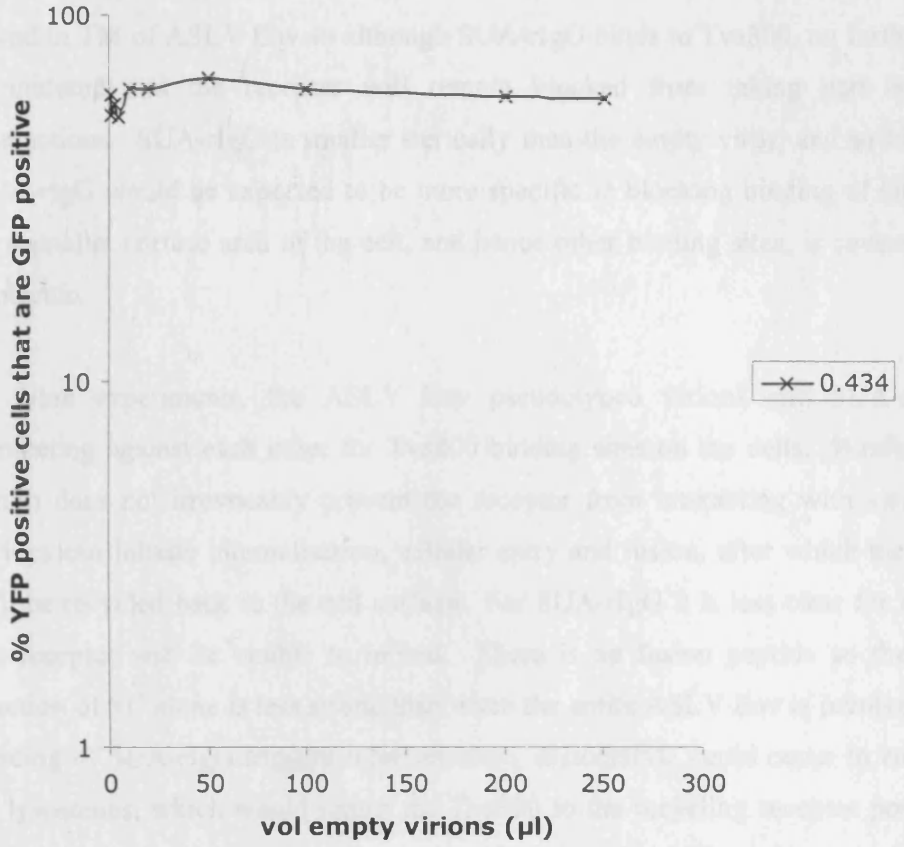


Fig. 4.6.2. Empty virions have little ability to compete out GFP virions for free Tva800 receptor in cells transduced with vector encoding HA-Tva800ATG, and are not effective in reducing infection. Cells were plated, transduced and infected as described in the legend to figure 4.6.1, except that Tva800 ATG was used. Lower MOI were assessed, but levels of YFP fluorescence were <1%, making analysis unreliable. Results from one of two experiments are shown.

However, as shown in figure 4.6.2, for cells transduced with HA-Tva800 ATG, increasing the level of empty virus did not decrease titres, even though cells were transduced with very low levels of the HA-Tva800 ATG vector (at MOI of 0.4). Around 50% of YFP expressing cells were infected with GFP vector at all volumes of empty virion used. This suggests that Tva800 is much more efficiently expressed from the vector with the wild-type start codon, as might be expected, and that when greater levels of Tva800 are present the empty virus has less of an inhibitory effect.

Another way to block binding of Tva800 receptor to ASLV Env is to use SUA-rIgG. This hybrid protein is made of SU of ASLV Env linked to rabbit immunoglobulin G.

The fusion peptide, which mediates the fusion of viral and cellular membranes, is found in TM of ASLV Env so although SUA-rIgG binds to Tva800, no further fusion is initiated and the receptor will remain blocked from taking part in further interactions. SUA-rIgG is smaller sterically than the empty virus, and so binding of SUA-rIgG would be expected to be more specific in blocking binding of GFP virus, as a smaller surface area of the cell, and hence other binding sites, is covered by the molecule.

In these experiments, the ASLV Env pseudotyped virions and SUA-rIgG are competing against each other for Tva800 binding sites on the cells. Binding of the virion does not irrevocably prevent the receptor from interacting with virus as the virions can initiate internalisation, cellular entry and fusion, after which the receptor will be recycled back to the cell surface. For SUA-rIgG it is less clear for how long the receptor will be unable to rebind. There is no fusion peptide so the binding reaction of SU alone is less strong than when the entire ASLV Env is involved. If the binding of SUA-rIgG triggers internalisation, dissociation could occur in endosomes or lysosomes, which would return the Tva800 to the recycling receptor pool. If no internalisation is triggered, however, the Tva800-SUA-rIgG could remain bound at the cell surface.

Mus dunni cells were transduced with Tva800 ATG or Tva800 AGG, and subsequently challenged with the fixed volume of 10µl GFP virus, mixed with increasing volumes of SUA-rIgG, made up to a fixed volume of 510µl in PBS.

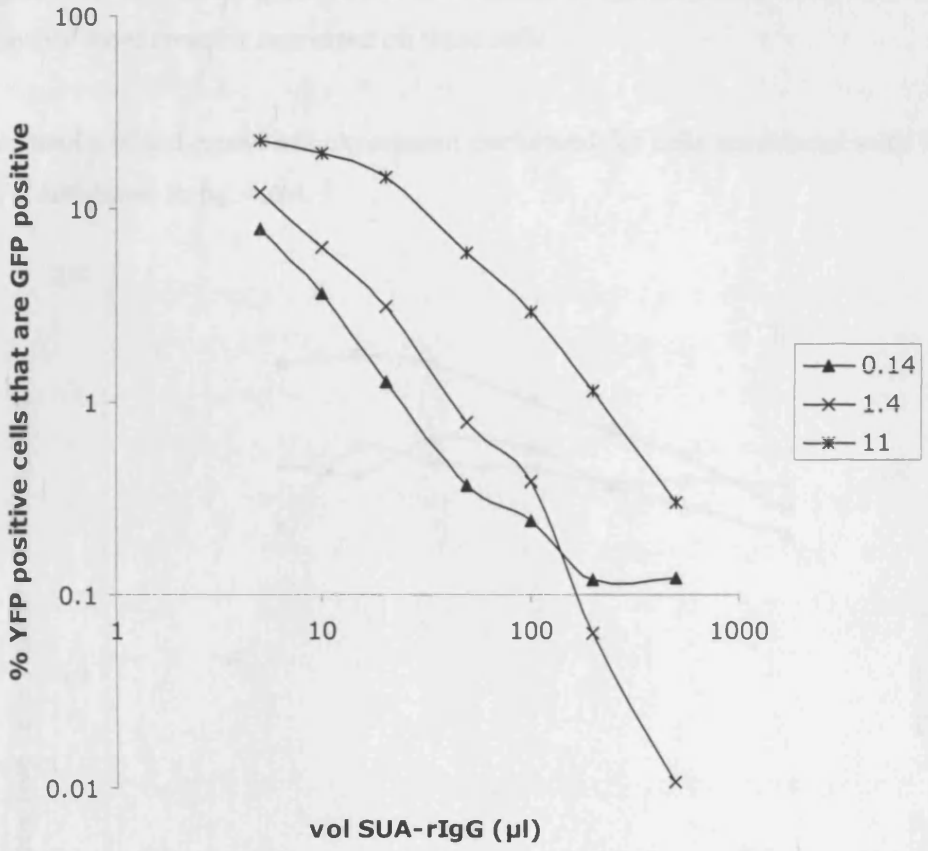


Fig. 4.6.3 Infection mediated by ASLV Env is inhibited by SUA-rIgG on cells transduced with Tva800AGG. *Mus dunni* cells were plated at $5x10^4$ cells per well and transduced with Tva800 AGG at MOI 0.1-11. Cells were then challenged with 10µl ASLV Env pseudotyped NB-MLV GFP vector mixed with 0-500µl SUA-rIgG containing-supernatant. The percentage of cells successfully infected with GFP vector was assessed after three days by FACS. Representative results from one of three experiments are shown.

The data in fig. 4.6.3 were obtained for cells transduced with Tva800 AGG, where receptor levels would be expected to be limited. Increasing the volume of SUA-rIgG has an increasingly inhibitory effect on the ability of a constant volume (10µl) of ASLV Env pseudotyped virus to enter cells. The decrease seen is equivalent for cells transduced with Tva800AGG at MOI 0.14 and 1.4, as in practice these MOI would both lead to cells being transduced with either one virus or none (in the case of an MOI of 1.4 is is simply that the proportion transduced with one virus will be higher), and hence cells that are expressing receptor will be expressing the same level. If cells are transduced at MOI 11 the inhibition remains considerable, although the curve

begins with a short lag period of non-inhibition (≤20µl), indicating that there is probably more receptor expressed on these cells.

The results of the equivalent experiment performed for cells transduced with Tva800 ATG are shown in fig. 4.6.4.

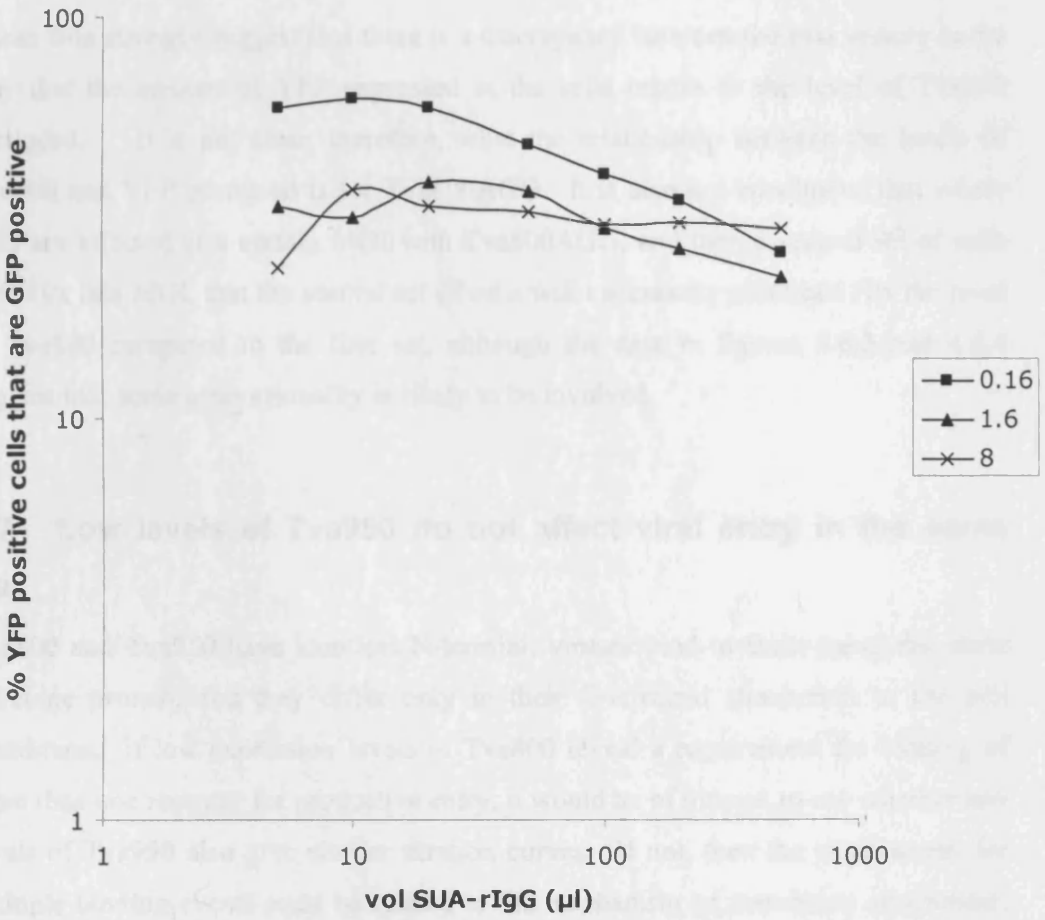


Fig. 4.6.4 Infection by GFP vector is much less inhibited by SUA-rIgG on cells transduced with Tva800ATG. *Mus dunni* cells were plated and transduced with Tva800ATG at MOI 0.16-8, after which infections were carried out as described in the legend to figure 4.6.3. The experiment was performed four times, results from one experiment are shown.

The curves obtained from the data presented in figure 4.6.4 are almost horizontal, indicating that the presence of SUA-rIgG is not inhibiting binding of ASLV Env pseudotyped GFP vector. A decrease in GFP expression is still seen for cells transduced with Tva800ATG at MOI 0.16. The percentage of cells expressing GFP decreases from 60% to 25% when the ratio of GFP vector: SUA-rIgG increases from

1:1 to 1:50, a decrease of 2.4-fold. However, for cells transduced with Tva800AGG the decrease is over 70-fold at all MOIs tested, which is clearly much more dramatic. When higher MOIs of Tva800ATG were tested (1.6 and 8), no decrease was seen, indicating that sufficiently high levels of Tva800 are produced that binding and entry of virus is uninhibited by SUA-rIgG.

These data strongly suggest that there is a discrepancy between the two vectors in the way that the amount of YFP expressed in the cells relates to the level of Tva800 produced. It is not clear, therefore, what the relationship between the levels of Tva800 and YFP produced is for Tva800AGG. It is also not conclusive that where cells are infected at a certain MOI with Tva800AGG, and then a second set of cells with 10x this MOI, that the second set of cells will necessarily produced 10x the level of Tva800 compared to the first set, although the data in figures 4.6.3 and 4.6.4 suggest that some proportionality is likely to be involved.

4.7 Low levels of Tva950 do not affect viral entry in the same way

Tva800 and Tva950 have identical N-termini, viruses bind to them using the same envelope protein, and they differ only in their C-terminal attachment to the cell membrane. If low expression levels of Tva800 reveal a requirement for binding of more than one receptor for productive entry, it would be of interest to see whether low levels of Tva950 also give similar titration curves. If not, then the requirement for multiple binding events must be related to the mechanism of membrane attachment. A point mutation was inserted into the vector pLgateway950IRESYFP at the start codon of Tva950 to give an AGG triplet (Tva950AGG). This was then transduced into *Mus dunni* cells, and successfully transduced cells detected by YFP expression. Titres of ASLV Env pseudotyped NB-MLV were then compared to cells transduced with the non-mutant Tva950ATG vector. The results are shown in fig 4.7.1.

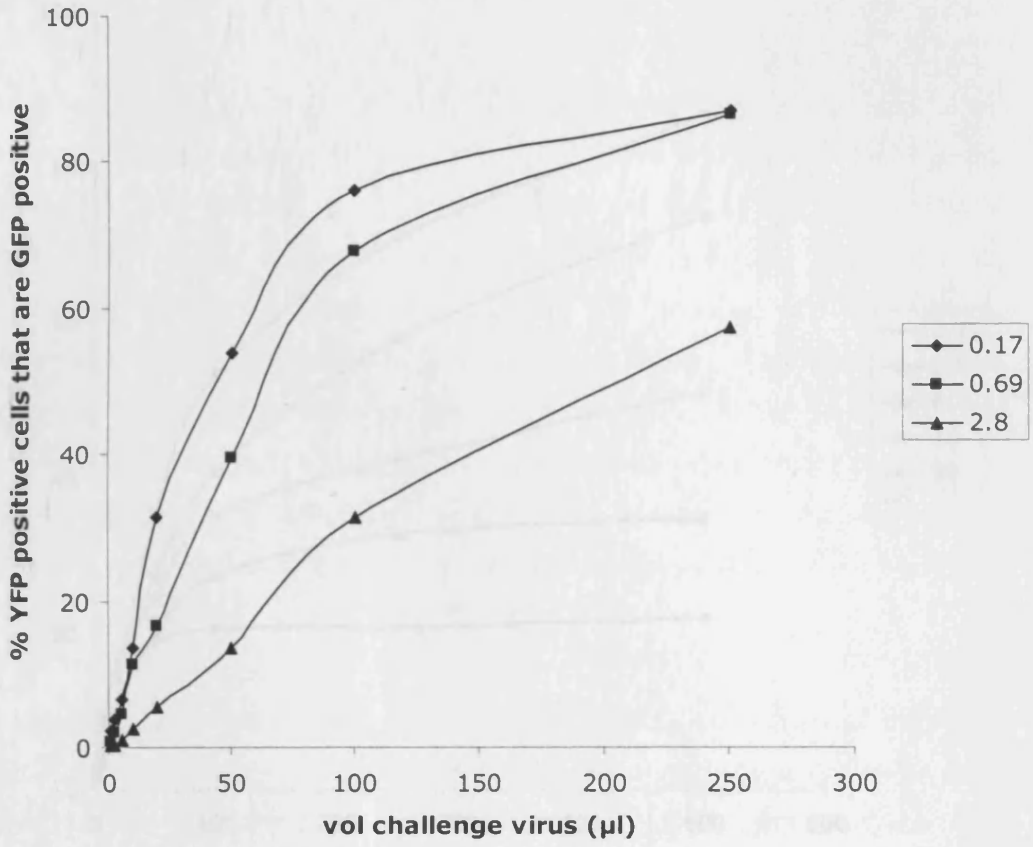


Fig 4.7.1 Increasing the MOI of Tva950ATG vector on cells does not change the shape of the titration curves obtained from application of increasing volumes of ASLV-Env/NB-MLV onto those cells. *Mus dunni* cells were plated and transduced with Tva950ATG vector at MOI 0.17-2.8. Cells were split and challenged after 3 days with ASLV-Env pseudotyped NB-MLV-encoding eGFP, and the percentage of cells infected analysed by FACS. Results shown are from one experiment of three performed.

When cells were transduced with low MOI Tva950ATG vector, there does not appear to be any limitation to viral entry at high titres of challenge virus, indicating that there are high levels of free receptor on the cell surface that are able to bind the high levels of incoming virus. In fact, when cells are transduced with higher MOI of Tva950ATG it appears that there is insufficient virus to infect all the cells that potentially could be infected, i.e. are expressing Tva950 (and YFP).

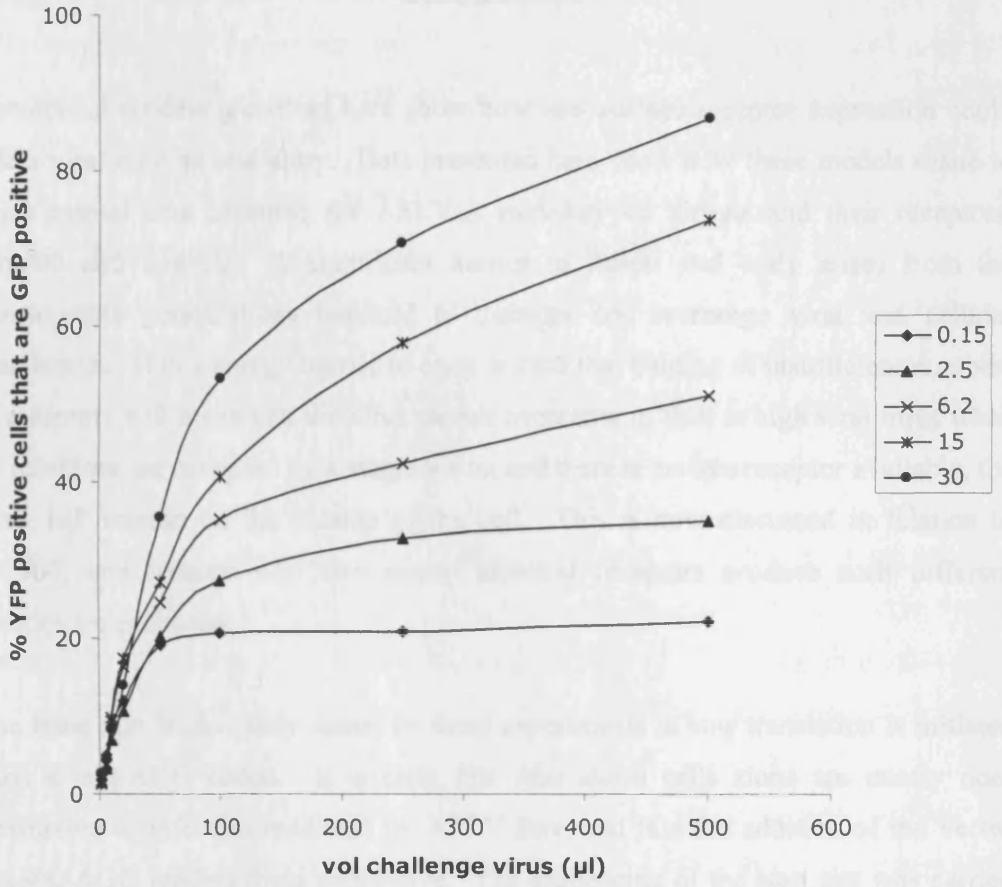


Fig. 4.7.2 Increasing MOI of Tva950AGG also does not change the shape of the titration curves obtained from ASLV Env pseudotyped NB-MLV. *Mus dunni* cells were plated and transduced with Tva950AGG at MOI between 0.15 to approximately 30, challenged and analysed as described in the legend to figure 4.7.1. Results shown are combined from one experiment with two separate Tva950AGG clones.

When the mutated vector Tva950AGG was used and titres of challenge virus were over 20µl a steady further increase was seen. Titres do not reach 100% for cells transduced with vector at MOI below 15% probably because of the high number of cells that are YFP positive but not receptor positive. If this graph is compared to the models shown in fig 4.3, the curves show the most similarity to those seen when it is modelled that binding of 1 receptor is sufficient for entry, although the fit is not as close as that for Tva800 and the 2-receptor model. If it is borne in mind that virions entering via Tva800 need to bind more than one receptor, probably two, for successful entry (fig. 4.2), this would indicate a major difference in mechanistic steps between Tva800 and Tva950 subsequent to binding of ASLV Env.

Discussion

Theoretical models presented here show how low surface receptor expression could affect viral binding and entry. Data presented here show how these models relate to experimental data obtained for ASLV-A pseudotyped virions and their receptors, Tva800 and Tva950. A significant barrier to fusion and entry arises from the considerable permutations required to disorder and rearrange viral and cellular membranes. If this energy barrier to entry is such that binding of insufficient numbers of receptors will mean that the virus cannot overcome it, then at high viral titres when all receptors are occupied by a single virion and there is no free receptor available, the virus will remain on the outside of the cell. This is now discussed in relation to Tva800, and reasons why two nearly identical receptors produce such different kinetics are proposed.

One issue that is obviously raised by these experiments is how translation is initiated from a non-AUG codon. It is clear that *Mus dunni* cells alone are utterly non-permissive to infection mediated by ASLV Env, and that the addition of the vector Tva800 AGG renders them permissive. The sequencing of the start site was carried out four times by in-house and commercial sequencing methods, and unambiguously found to be indeed AGG. Close examination of sequences in the vector that lie upstream of the start site did not reveal any in-frame start sites, and indeed there were several in-frame stop sites within 200bp. Interestingly, the mutation of T to G in the start codon would form a splice acceptor site (CACC**AGG**) but as any upstream start sites between the major splice donor site (just downstream of the primer binding site) and this site would be spliced out it is not likely that this is the reason why translation occurs. Start sites removed would include the major *gag* and *glyco-gag* initiation sites. Non-canonical start codons are by no means unknown, but are more usually found in plant genomes, bacteria, yeast, and viruses, and initiation is also much weaker than from AUG (Kozak 1983; Gren 1984; Cherpillod *et al.* 2004; Depeiges *et al.* 2006). Usage of these sites is very dependent on other sequences surrounding the altered start site (Chen *et al.* 2008; Wegrzyn *et al.* 2008). In one study of initiator codons of the mouse dihydrofolate reductase gene a total of nine variants were considered, where each variant differs from AUG by only one nucleotide (Peabody

1989). Each combination was able to direct synthesis of an apparently normal protein with some level of efficiency, except the codons AAG and AGG, and used the methionine initiator codon. Despite the base mismatch, the level of initiation from the codon ACG reached 10-15% that from AUG; this is much higher than the level of mismatch permitted during elongation and must represent some level of permissivity peculiar to the start site, as only $tRNA_i^{Met}$ is able to gain access. Thus while it is surprising that Tva800 protein is produced from the AGG codon, it is not completely beyond the realms of possibility. Very little protein will be produced, which forms part of the basis of the explanation of the odd-shaped titration curves obtained using the Tva800 AGG vector.

The next question that must be considered is how similar or different the mechanisms of the two receptors, Tva800 and Tva950, really are. In chapter 3 the routes of entry mediated by these two receptors were discussed. Here, the possibility is raised that the lipid raft location of Tva800 has a significant effect on the binding requirements for entry. A comparison of the graphs in figures 4.2 (Tva800), 4.7.1 and 4.7.2 (Tva950) with the theoretical models shown in figure 4.3 would suggest that there is a significant difference between the two. The graphs for Tva800 appear to resemble the models for which >1, probably 2 receptors need to be bound for entry. For Tva950, however, the percentage of virus infecting the cells increases as the volume of viruses increases. There is no significant change in the shape of the titration curve seen as the level of Tva950 in the cells increases, suggesting that the presence of an increased number of free Tva950 receptors has no effect on the ability of ASLV Env pseudotyped virions to enter the cell. This strongly suggests that a single free Tva950 receptor is sufficient for the virus to enter the cell.

The two forms of the receptor differ only in their attachment to the membrane and the localised milieu of the plasma membrane (Tva800 resides in lipid rafts (Narayan *et al.* 2003), and one or both of these clearly have a profound effect on subsequent steps of viral entry. Data presented in chapter 3 would strongly suggest that the situation of Tva800 in lipid rafts, in contrast to Tva950 which is found outside, is responsible for the stability of bound virus under an NH_4Cl-induced block. As described in the introduction, lipid rafts are areas of high cholesterol, and tight packing of lipids and fatty acyl chains, and the structure is more akin to a gel than the rest of the membrane,

which resembles energetically the state of a liquid. As discussed in chapter 3, these microenvironments are generally preserved after internalisation of areas of the plasma membrane. These data presented here would further suggest that this difference in localisation of the receptor at the cell surface has a profound effect on downstream events, not only on the stability of the receptor under NH_4Cl in an intracellular compartment, but also on the activation energy level required to overcome the barrier to induce fusion between the viral and cellular membranes. If binding of more than one Tva800 receptor is necessary for entry then it would suggest that there is a higher activation energy to overcome the barrier to fusion within lipid rafts; the tighter association necessary from the extra binding, and additional energy provided by more than one fusion protein and receptor driving the reaction process, is necessary to overcome this. Plasma membrane proteins attached via a GPI anchor are only associated with the outer leaflet of the plasma membrane whereas the anchor of transmembrane proteins (by very definition) span both. It is feasible that causing the perturbations to the cell membrane necessary for fusion of viral and cellular membranes, and hence viral entry, is easier when the receptor spans both leaflets, as is the case for Tva950. It is also possible that this is linked to the fact that as Tva800 is not anchored to both leaflets, it is more prone to being extruded from the cell membrane due to the force exerted on it subsequent to virus binding, in comparison to Tva950.

These data and this hypothesis would fit in well with time course data by Lim et al., who calculated that the initial stages of entry (to the point of fusion) via Tva800 took 2-3 times longer than when Tva950 was used, but entry overall was just as efficient (Lim et al. 2004). This is not an increase in timescale likely to be significantly detrimental to the overall probability of success of the infectious cycle of the virus, especially if having an alternative route of entry into the cell might render those cells permissive to the virus.

For the cell there are very real advantages in organising the cell membrane so that clustering of certain types of receptor into small regions bring them into close proximity, and so it is hardly surprising that pathogens have evolved to use these microdomains to their advantage. Lipid rafts create platforms for sorting and signal transduction, the latter of which is particularly relevant in cells of the immune system

where colocalisation of receptors can concentrate signals and generate positive feedback, resulting in amplification. There may be significant advantages to a virus in using raft-associated proteins to enter cells, as this then directs the virus into certain sorting pathways, or could provide colocalisation of receptors and coreceptors into the same region of the plasma membrane, although this is not the case for ASLV, as no coreceptor is used. The current physiological significance of the presence of both types of receptor is not currently known. For ASLV, it could be an advantage to be able to use either Tva800 or Tva950, depending on which species or cell type is targeted. It is also possible that being able to use one could be an irrelevant by-product of being able to use the other.

Overall it can be concluded from the data presented here that low levels of viral receptor expression can prevent virus being able to enter cells when a high titre of virus is used, and the virus needs to bind more than one receptor in order to be able to enter cells. This is not a particularly novel concept in either virology or retrovirology. The rabies glycoprotein is organised into trimers and mediates binding and fusion. An estimate of the number of these glycoproteins involved in fusion (after binding) has put the number between 13 and 19 (Roche and Gaudin 2002). In retrovirology, a mathematical analysis of binding and entry of HIV-1 via gp120 and CD4 indicated that while binding of one gp120 may be sufficient to mediate viral binding, the involvement of further gp120-CD4 pairs is necessary to mediate viral penetration (Layne et al. 1990). At low concentrations of inhibitory soluble CD4, when free gp120 is readily available on the virion, each gp120 acts independently in contributing to infection. Once concentrations of soluble CD4 are sufficiently high to block about half of the gp120 sites, addition of further CD4 blocks infection synergistically, indicating that cooperative effects then come into play (Layne et al. 1990).

Studying these effects is important for applications involving gene therapy in clinical settings. Traditionally it has been assumed that a high viral titre is necessary in order for entry of proportions sufficient to achieve the desired effect. However, this assumption and strategy could be counter-productive if viral envelopes and receptor combinations with entry requirements such as ASLV Env and Tva800 are used, as excessive numbers of virions would actually inhibit entry rather than increase titres.

The simplicity of the ASLV Env/Tva800 system lies in the involvement of a conclusively defined receptor with no coreceptors. These would be significant advantages for a model system which could be used to further characterise cooperativity in viral entry.

Chapter 5

Characterising a Block to Infection in HeLa CD4 Cells

Trim5α was isolated and confirmed to act as a restriction factor in 2004 (Stremlau et al. 2004). It was first reported in 2000 that a block to infection similar to the restriction of N-MLV by $Fv1^b$ occurred in certain mammalian species (Towers *et al.* 2000). As Fv1 is found only in mouse cells, this was an indication that Fv1 could be the first of many potential restriction phenomena. Primate variants of Trim5α were eventually revealed as the causative agent of both HIV restriction in rhesus macaques (Stremlau et al. 2004), and N-MLV restriction in primate cell lines (Keckesova et al. 2004; Yap et al. 2004) and searches for other factors continue (Gao et al. 2002; Bruce et al. 2005).

Characteristic traits of Fv1 and Trim5α include that they can both be abrogated, and that the capsid sequence of the incoming virus determines whether or not it will be restricted; for example, N-MLV, but not B-MLV is restricted by Trim5α. Where restriction by Fv1 and Trim5α differ, however, is in the timing of the block to restriction. The stages of infection that the virus traverses can be followed by assessing the products of reverse transcription. Restricted virus entering Fv1 positive cells completes reverse transcription, as shown by equal levels of reverse transcription products in restrictive and non-restrictive cells, and accumulation of 2LTR circles in the nucleus (Jolicoeur and Rassart 1980; Yang et al. 1980). In Trim5α positive cells, however, the blockage is before reverse transcription (Munk *et al.* 2002; Stremlau *et al.* 2004). It is not currently known with any precision what the mechanisms of Fv1 and Trim5α are, whether similar mechanisms with small perturbations cause the difference in timing, or if the modes of action are altogether different.

Now that it has been demonstrated that Fv1 is much less a unique oddity than a trend-setter, and with the suggestion that discovery of further restriction factors could have therapeutic value, there has been interest in scrutinising cell types from a variety of species for signs of similar innate (or artificially induced) activity (Gao and Goff 1999; Bruce et al. 2005). Cells that exhibit resistance to infection under certain

circumstances have been discovered both by serendipity and rigorous screens, and novel genes uncovered that induce resistance (Gao and Goff 1999; Bruce et al. 2005; Pineda et al. 2007). The Trim family has come under particular scrutiny. Of the >70 members of this family (Reymond et al. 2001), many currently have no known or essential function in the cell, and it has been suggested that while they initially arose through gene duplication (Towers 2007), many may have persisted due to antiviral activity conferred. Evidence of protective functions of Trims other than Trims 5 (Stremlau et al. 2004) and 1 (Yap et al. 2004) is gradually being uncovered, as well as variants of Trim5 itself in which CypA replaces the B30.2 region (Sayah *et al.* 2004; Newman *et al.* 2008; Wilson *et al.* 2008). A screen of 36 human Trim proteins revealed some that actually appeared to enhance viral release (Trims 25, 31 and 62), and several that had inhibitory effects also acted late in the replicative cycle (Uchil et al. 2008). Other Trims appear to first need activation, for example Trim22 is upregulated by the interferon response, and inhibits HIV-1 particle release (Barr et al. 2008).

In chapter 3 it was shown that Fv1 and Trim5α-mediated restrictions to infection are not affected by the route that the virus takes into the cell, and it was hypothesised that this might generally be true for other restriction factors as virions have to pass through the cytoplasm. In 2004 a new block to replication of HIV-2 was described, and characterised as having both capsid and envelope dependent components (Schmitz et al. 2004). The capsid sequence of the virus altered susceptibility to Lv2, so it was suggested that it could be caused by a restriction factor like Fv1 or Trim5α, although inhibition of Trim5α activity did not increase replication in restrictive cells. However, there was also a degree of envelope dependence since a virus with a susceptible capsid sequence could still replicate if it was pseudotyped by VSV-G envelope protein, which directs the virus into a pH-dependent endocytic route of entry. This envelope dependence, along with the separation of the effect from Ref1 activity, suggested that a new restriction had been uncovered, and it was named Lv2 (lentiviral restriction factor 2). The change in Lv2 activity seen when different envelope proteins were used, taking the virus into the cell by different routes, implies that the nature of intracellular compartments, with all the associated diversities in pH, proteases, proteins, the structure of the compartment and other components, might have a profound effect on the ability of a virus to replicate successfully.

Lv2 was characterised in four cell types; HeLa CD4 cells (Scherer et al. 1953; Maddon et al. 1986) and Ghost/CXCR4 (Cecilia et al. 1998) restrict Lv2-susceptible viruses, whereas NP2*/CD4/CXCR4 (NP2*) (Soda et al. 1999; Schmitz et al. 2004; Marchant 2006) and U87*/CD4/CXCR4 (U87*) (Bjorndal et al. 1997) do not. This means that VSV-G pseudotypes can replicate in all cell types, but that virions with an Lv2-susceptible envelope can only replicate successfully in U87* or NP2* (Lv2-negative) cells. The phenomenon of Lv2 was initially spotted by investigation of a primary isolate (prCBL-23) and a T-cell line adapted isolate (CBL-23), but studies were continued using their equivalent molecular clones known as MCR and MCN, molecular clone restricted (Lv2 sensitive) and non-restricted (Lv2 insensitive) respectively (Schmitz et al. 2004). These names refer to the whole clone, Env and Gag-pol together.

When the Gag-pol and Env of MCR and MCN were swapped, the different contributions of Env and Gag were assessed (Schmitz et al. 2004; Reuter et al. 2005). Virions composed of MCR Gag-pol and Env replicated to between 50 to 60-fold higher titres on U87* cells compared to HeLa CD4 (Schmitz et al. 2004) or restrictive Ghost/X4 cells (Reuter et al. 2005). MCR Env pseudotypes conferred 20-fold restriction on MCN Gag (Schmitz et al. 2004), with an additional restriction to infection (19-fold (Reuter et al. 2005) or 5-fold (Schmitz et al. 2004)) in HeLa CD4 cells compared to U87* cells attributable to Gag. Production of virus from several different cell types had no effect on susceptibility to Lv2 (Schmitz et al. 2004). Env and Gag act in concert to determine the extent to which the virus is restricted. The determinant in Gag is in CA, at position 207 where there is an isoleucine (I) and valine (V) seen in MCR and MCN respectively (Schmitz et al. 2004). The Env determinant was mapped to position 74 in SU; glutamic acid (E) for MCR and glycine (G) for MCN (Reuter et al. 2005).

The restriction activity of Lv2 does not require an HIV-2 core to be effective; MCR Env pseudotyped 8.91 core (i.e. HIV-1) plated on HeLa CD4 cells give low titres equivalent to MCR core (Marchant et al. 2005), showing that Env is a key determinant and can confer sensitivity on an unrelated core. Therefore throughout

this chapter experiments were carried out with HIV-1 (8.91) cores, and HIV denotes HIV-1 unless otherwise stated.

Additional studies also characterised more precisely the nature of the cellular pathways by which Lv2 restriction operates. As VSV-G pseudotypes of MCR were successful in replicating, the conclusion was drawn that entry via an endocytic route to both Lv2-negative and Lv2-positive cells is productive for the virus, and permits it access to the nucleus subsequent to entry and reverse transcription (Schmitz et al. 2004). Fusion via CD4 and CXCR4 in cells where Lv2 is operative, on the other hand delivers the virus into a cellular compartment where Lv2 is active and able to suppress infection. After manipulation of cellular trafficking routes using NH_4Cl, bafilomycin A1 (prevents the acidification of endosomes), hypertonic sucrose (inhibits endocytosis) and β-methylcyclodextrin (depletes membrane cholesterol) it was concluded that Lv2 is active after delivery via a lipid-raft dependent, pH-independent endocytic route, which requires membrane cholesterol (Marchant et al. 2005). A summary of these criteria is shown in the following diagram.

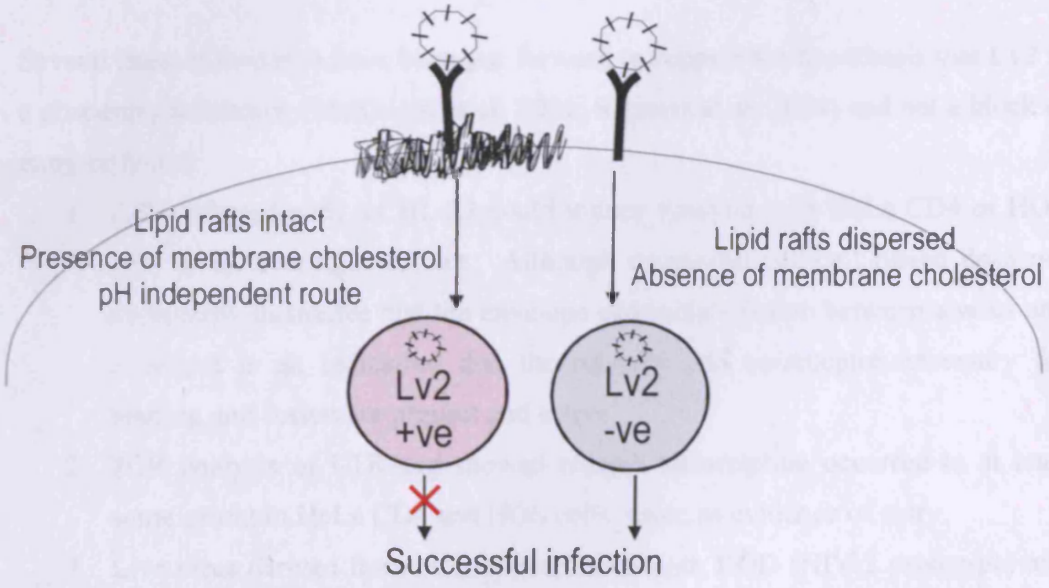


Figure 5 A schematic showing two potential entry pathways for MCR-pseudotyped virions entering Lv2-positive cells.

As the coreceptor CXCR4 is required, presumably this plays a role in triggering fusion along this pathway (McKnight *et al.* 1998). If these pathways are inhibited by the chemicals mentioned above, restriction by Lv2 is reduced, and replication of MCR on HeLa CD4 cells is as productive as MCN. Although NH_4Cl rescues infection, it

was determined that this is not related to the inhibition of lysosomal degradation, as similar experiments with bafilomcin A1 which also prevents acidification of endosomes and lysosomes had no effect (Marchant et al. 2005). All of these treatments had no significant effect on infection by MCN.

An indication of whether restriction is dominant or recessive can be drawn from experiments in which heterokaryons of restrictive and susceptible cells are made, and then infected (Cowan et al. 2002). Successful infection indicates a recessive factor, whereas infection levels similar to those seen in restrictive cells alone would have indicated a dominant factor. Such experiments with Lv2-positive and negative cells, however, did not give conclusive results, indicating that the integrity of the plasma membrane (which is seriously comprised during pertubations to create heterokaryons) is necessary for Lv2 restriction (Schmitz et al. 2004). Alternatively, if the block to infection can be abrogated, that is, overcome with high titres of susceptible virus, then this indicates that the block is caused by a dominant, saturable factor. Abrogation of Lv2 was possible to a very limited extent; incubation with restricted virus raised titres by between 3- and 6-fold (Marchant 2006).

Several lines of evidence have been put forward to support the hypothesis that Lv2 is a post-entry restriction (McKnight et al. 2001; Schmitz et al. 2004) and not a block at entry or fusion:

1. Cells infected with prCBL-23 could induce syncytia with HeLa CD4 or HOS cells in an overnight culture. Although successful cell-cell fusion does not necessarily guarantee that the envelope can initiate fusion between a virus and a cell, it is an indication that the receptor and co-receptor necessary for binding and fusion are present and active.
2. PCR analysis of LTR-gag showed reverse transcription occurred to at least some extent in HeLa CD4 and HOS cells, taken as evidence of entry.
3. Live virus derived from cells infected with both ROD (HIV-2 prototype) and prCBL-23 generate hybrid particles with two envelopes. These virions replicate to high titre on HeLa CD4 cells. If antibody is added to block entry by ROD Env, titres are not reduced, and are equivalent to the infectivity of the co-pseudotyped virus alone with no antibody, indicating that the prCBL-23 Env is fusion-competent.

4. qPCR data by Schmitz et al. are interpreted to support the hypothesis that MCR and MCN are both equivalent in infectious capacity and ability to infect cells, and that the block to infection by MCR must therefore be post-reverse transcription (Schmitz et al. 2004). These data are discussed at length in section 5.5.

These then, were the conclusions from previous studies of the restrictive nature of HeLa CD4 cells to infection by MCR pseudotypes. If the block is post-entry, then the question arises as to where, when, and how the block manifests itself. Schmitz *et al.* suggest that the virus is being directed into an intracellular compartment with no access to the nucleus. Taking these studies further would reveal much about viral entry, and the nature of intracellular compartments. If Lv2 represents a novel mechanism of restriction, it would represent a significant exception to the apparent non-route of entry dependence of other restriction factors investigated so far and discussed in chapter 3. In this chapter, I describe work done to try and further understand this block to retroviral infection.

Results

5.1 Replication of MCR Env pseudotyped HIV and NB-MLV in HeLa CD4 cells is inhibited in HeLa CD4 cells but not in NP2* cells

Previous experiments comparing titres of MCR, MCN and VSV-G on HeLa CD4 and U87* or NP2* cells were done by counting foci of infection. Analysis by FACS is a less user-subjective method that also allows analysis of many more cells, so an experiment was carried out to assess the relative infectivities of VSV-G and MCR pseudotypes on Lv2-positive and Lv2-negative cells as assayed by FACS. This would also permit verification of a basic premise of Lv2 restriction, that MCR Env pseudotypes are competent for the early stages of replication in some cell types but not in others. Both cell types should be permissive to VSV-G pseudotypes.

The permissive (Lv2-negative) U87* cell line was used in previous reports to demonstrate the functionality of MCR Env pseudotyped HIV cores. However, these cells could not be used in our assays, as there was a small but persistent level of auto-

fluorescence in the GFP channel that interfered with fluorescent cell sorting (not shown). Instead the equally permissive human glioma cell line NP2* was used for FACS assays. $5x10^4$ cells of HeLa CD4 and NP2* cells were plated and infected with 5-400µl of VSV-G or MCR pseudotyped HIV or NB-MLV cores.

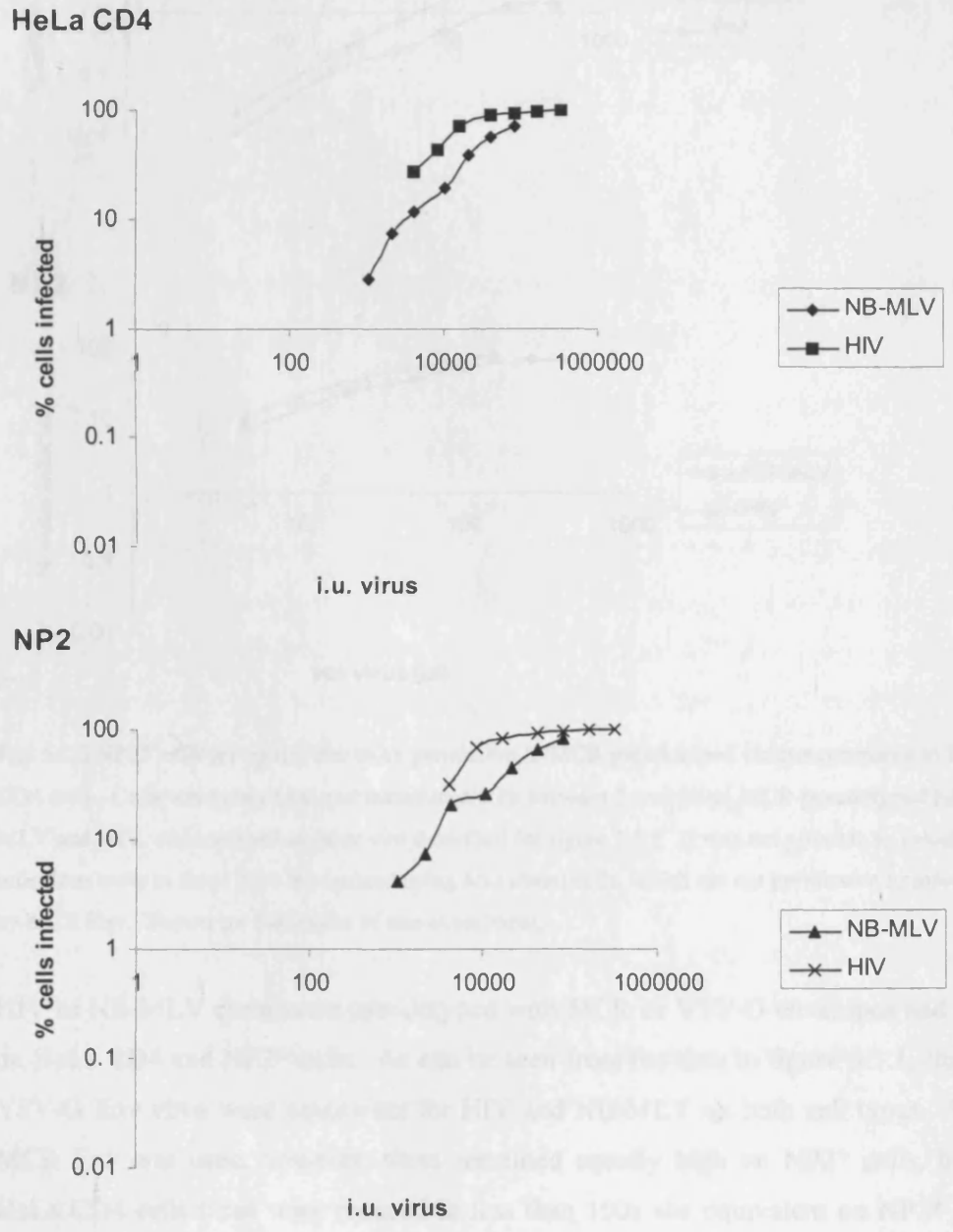


Fig 5.1.1 VSV-G pseudotyped HIV-1 and NB-MLV cores replicate to equally high titres on HeLa CD4 and NP2* cells. $5x10^4$ cells were plated and titrated with between 5-400µl of VSV-G pseudotyped HIV-1 or NB-MLV (graphs are plotted with infectious units as measured on *Mus dunni* cells, i.u., for ease of comparison). Analysis was 3-days later by FACS. Shown are the typical results of one experiment.

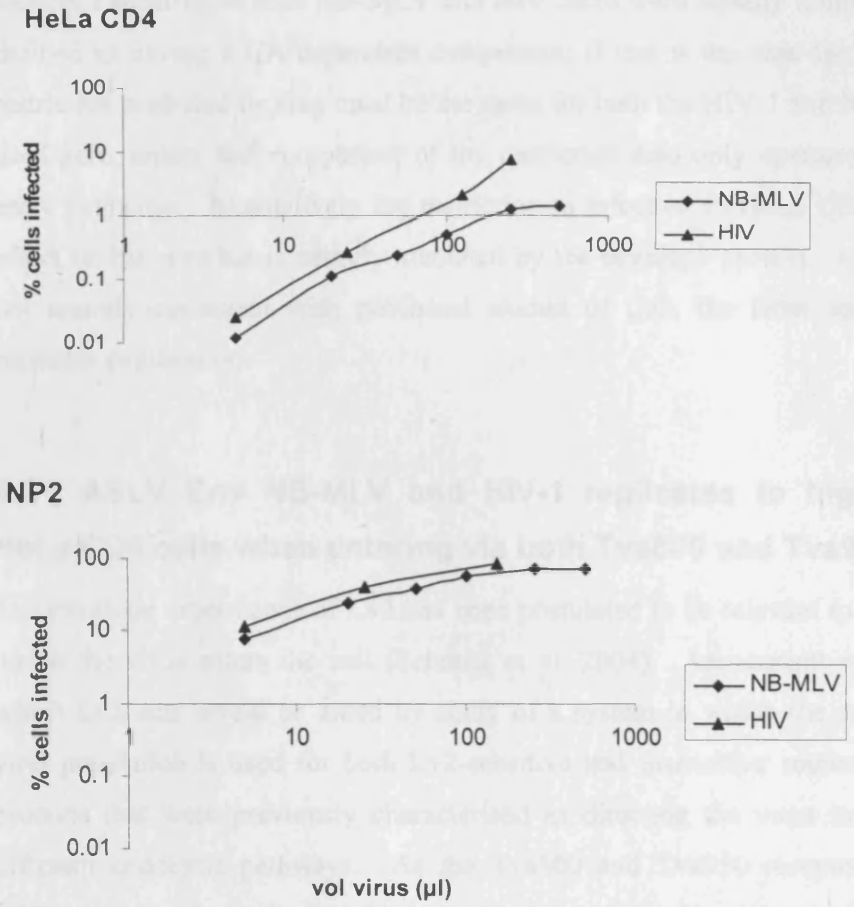


Fig. 5.1.2 NP2* cells are up to 100x more permissive to MCR pseudotyped virions compared to HeLa CD4 cells. Cells were plated out and transduced with between 5 and 500ul MCR pseudotyped NB-MLV and HIV, and analysed as otherwise described for figure 5.1.1. It was not possible to calculate infectious units as these were normalised using *Mus dunni* cells, which are not permissive to infection by MCR Env. Shown are the results of one experiment.

HIV or NB-MLV cores were pseudotyped with MCR or VSV-G envelopes and titred on HeLa CD4 and NP2* cells. As can be seen from the data in figure 5.1.1, titres of VSV-G Env virus were equivalent for HIV and NB-MLV on both cell types. When MCR Env was used, however, titres remained equally high on NP2* cells, but on HeLa CD4 cells titres were reduced to less than 100x the equivalent on NP2* cells, reaching under 10% even with a very high virus inoculum. This is a bigger ratio even than the 50 to 60-fold difference previously reported (Schmitz *et al.* 2004). This difference indicates that there is a block at some stage of the replicative cycle of MCR Env pseudotyped cores that prevents them from infecting these cells. Further tests are needed to establish where this block lies. Interestingly the block is clearly not CA-

sequence specific, as both NB-MLV and HIV cores were equally inhibited. Lv2 was defined as having a CA-dependent component; if this is the case the component of restriction mediated by Gag must be the same for both the HIV-1 and NB-MLV cores used here, unless this component of the restriction also only operates down certain entry pathways. Alternatively the restriction to infection in HeLa CD4 cells has no effect on the core but is entirely mediated by the envelope protein. Although this is not entirely consistent with published studies of Lv2, the latter seems the more probable explanation.

5.2 ASLV Env NB-MLV and HIV-1 replicates to high titres on HeLa CD4 cells when entering via both Tva800 and Tva950

The envelope dependence of Lv2 has been postulated to be relevant to the manner in which the virus enters the cell (Schmitz et al. 2004). Assessment of the route by which Lv2 acts would be aided by study of a system in which the same infectious viral population is used for both Lv2-sensitive and insensitive routes, and receptor proteins that were previously characterised as directing the virus into the cell by different endocytic pathways. As the Tva800 and Tva950 receptors specifically fulfilled these criteria, it was of interest to see if a virus that entered via either one of the receptors expressed in HeLa CD4 cells would encounter Lv2-type restriction or not. Entry via Tva800 would be additionally interesting as it is a GPI-linked receptor, and resides in lipid rafts, which are important for Lv2-sensitive entry. Studies of Lv2 would be greatly enhanced if the same virus could be used for assessment of different pathways, rather than using different viral envelopes. To assess their permissivity, HeLa CD4 cells were transduced with high volumes of vector encoding Tva800 or Tva950, and three days later challenged with ASLV Env pseudotyped NB-MLV.

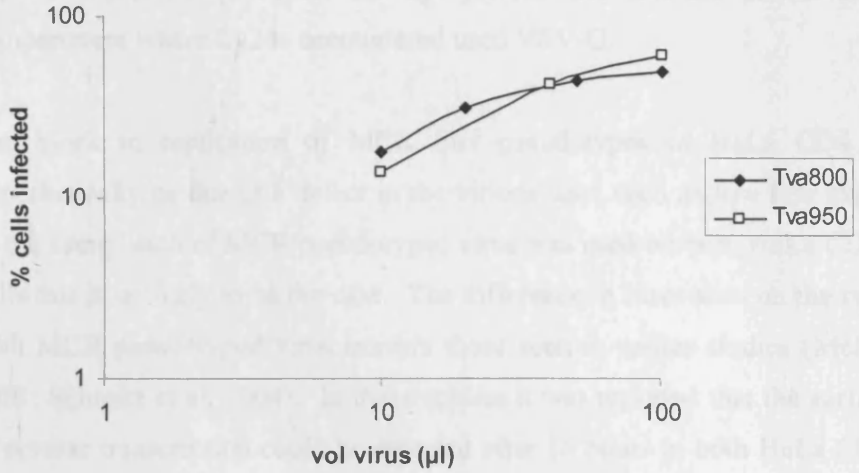


Fig. 5.2 HeLa CD4 cells expressing Tva800 and Tva950 can be successfully infected with ASLV Env pseudotyped virions. $5x10^4$ cells were plated and transduced with Tva800 or Tva950 vector, then challenged with 1-200µl ASLV Env pseudotyped NB-MLV. Analysis was by FACS. Shown are the results of one experiment with percentages of GFP positive cells as a function of all cells rather than just the cells expressing the receptors. The YFP fluorescence was faint and the receptor/YFP-positive population hard to separate, hence titres do not approach 100%.

Fig. 5.2 shows that virus enters and replicates successfully in HeLa CD4 cells after entering via either Tva800 or Tva950. There is hence no difference shown between these two routes of entry. There are many other routes into the cell, however, and this experiment does not preclude the possibility that Lv2 may operate down a third route, separate from those of Tva800 and Tva950.

Usage of MCN Env did not give consistent results either as rendering a virus Lv2 sensitive or insensitive. In HeLa CD4, NP2* and U87* cells virus pseudotyped with MCN was consistently unable to infect >5%, even at high viral titres (E. Gray and M. Yap, data not shown). It is not entirely clear why this is the case, given that the laboratory where it was cloned has used it to pseudotype a variety of viral cores successfully (Schmitz *et al.* 2004). 293T cells were used in virus production both previously and for experiments described here, so a difference in efficiency of production is unlikely to be the case. MCN differs from MCR by 7 amino acids in SU, and also at the C-terminus, as MCN has an extra 118 amino acids (Reuter *et al.* 2005). Ideally an investigation into MCR would use MCN as a counterpart, but because it was not possible to use MCN to generate viable virus in our lab, further

experiments that required an envelope protein that does not deliver the virus to a compartment where Lv2 is encountered used VSV-G.

The block to replication of MCR Env pseudotypes in HeLa CD4 cells could hypothetically be due to a defect in the virions used such as low Env expression, but as the same batch of MCR pseudotyped virus was used on both HeLa CD4 and NP2* cells this is unlikely to be the case. The difference in titres seen on the two cell types with MCR pseudotyped virus mirrors those seen in earlier studies (McKnight et al. 2001; Schmitz et al. 2004). In these studies it was reported that the earliest products of reverse transcription could be detected after 18 hours in both HeLa CD4 cells and NP2* cells, which suggested to the authors that the virus was competent to both enter and reverse transcribe and that the block in HeLa CD4 cells is not due to an entry defect.

5.3 Titres of other CD4/CXCR4-tropic viruses envelopes are also significantly reduced in HeLa CD4 cells

In order to assess whether Lv2 uniquely affects MCR pseudotyped virions in HeLa CD4 cells, or whether another viral envelope that uses CD4 and CXCR4 as receptor and co-receptor also makes the virus susceptible to Lv2, NL4-3 Env, was used. 5-500µl virus made with HIV core and NL4-3 Env was titrated onto HeLa CD4 and NP2* cells.

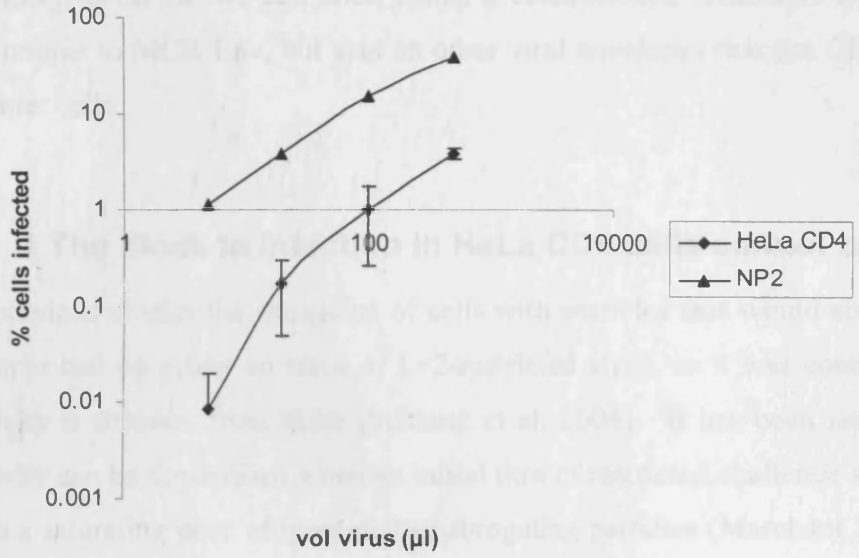


Fig. 5.3 Virus pseudotyped with NL4-3 envelope is not able to replicate to high titres on HeLa CD4 cells. $5x10^4$ HeLa CD4 and NP2* cells were plated out, and infected with between 5-500µl NL4-3 pseudotyped HIV. The infection was analysed 3 days later by FACS. Shown are the results of two experiments (for HeLa CD4 cells) and one for NP2* cells, error bars represent sdm.

These results show that if any volume of input NL4-3 Env pseudotyped virus is considered, NP2* cells are between 10- to 100-fold more permissive than HeLa CD4 cells. For example, when 500µl of virus was used, the percentages of HeLa CD4 and NP2* cells infected were 3.8% and 38.2% respectively, a difference of 10-fold. The difference was greater at lower viral titres. These results are in agreement with similar experiments conducted by Schmitz et al., but the interpretation of those data offered here will differ from theirs (Schmitz et al. 2004). From their data, HIV-1 cores pseudotyped either with MCR and NL4-3 envelopes reveal differences in titres of approximately 40- and 10-fold respectively. The conclusion drawn in (Schmitz et al. 2004) was that usage of NL4-3 envelope 'rescues the block to replication' compared to MCR Env. However, this conclusion cannot be supported for either their data or for these presented here. The 'rescue' in both cases is minor, of the order of 4-fold for their data (i.e. a reduction of a 40-fold to a 10-fold block to infection). For my data the difference between titres of MCR pseudotypes on HeLa CD4 and NP2* cells was between 40-50-fold, and therefore using NL4-3 Env made no significant difference. Whether data presented here or those from previous studies are considered, there remains at least a 10-fold difference between titres of NL4-3 Env

pseudotypes on the two cell lines, which is considerable. Therefore Lv2 restriction is not unique to MCR Env, but acts on other viral envelopes that use CD4 and CXCR4 to enter cells.

5.4 The block to infection in HeLa CD4 cells cannot be abrogated

In previous studies the saturation of cells with particles that would abrogate Fv1 and Trim5α had no effect on titres of Lv2-restricted virus, so it was concluded that Lv2 activity is separate from them (Schmitz et al. 2004). It has been reported that Lv2 activity can be diminished when an initial titre of restricted challenge virus is overlaid with a saturating dose of (potentially) abrogating particles (Marchant 2006), and both abrogation and challenge dose enter the cell simultaneously.

These previous abrogation experiments for Lv2 were carried out with initial application of the challenge dose at 4°C for 1hr, after which abrogating dose was applied for 30 min, as it was suggested that binding of the abrogation dose first could occlude receptors at the cell surface (Marchant 2006). An increase in titres of restricted virus of between 3- to 6-fold in HeLa CD4 cells was revealed. However, this moderate increase does not reflect the magnitude of the difference in titres between permissive and non-permissive cells (>60x), although it could be indicative of multiple blocks to replication. In experiments reported below, the abrogating dose was applied 3 hours prior to challenge, as previously described (Towers et al. 2002; Dodding et al. 2005), so that the potential saturability of the Lv2 factor(s) could be assessed in a direct comparison with that of Fv1 and Trim5α. The volume of abrogating virus used was equivalent to that used to saturate Fv1 and Trim5α (Towers *et al.* 2002; Dodding *et al.* 2005). Receptor recycling would be complete in the 3 hours that the cells spend at 37°C between abrogating and challenge doses.

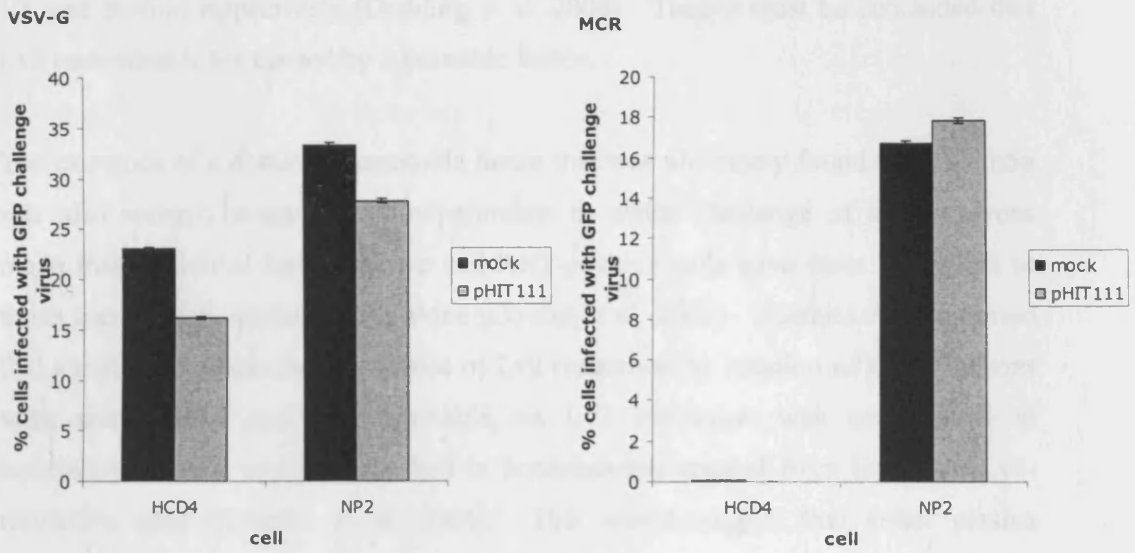


Fig 5.4 Pre-incubation of HeLa CD4 cells with restricted virus does not raise titres of a second, challenge dose of virus. Titres on NP2* cells are also unaffected, as are titres of VSV-G pseudotypes. The titres obtained from a dose of GFP vector-carrying challenge virus after pre-incubation with either 293T-cell extract (mock) or pHIT111 vector-carrying virus is shown for when both viral and challenge particles were pseudotyped with VSV-G or MCR Env. 500µl of a neat abrogation dose was overlaid onto $1x10^5$ cells plated 24 hours earlier, and incubated at 37°C for 3 hours. Cells were then washed gently with PBS, and challenged with fixed volumes of GFP challenge virus. Titres were determined 3 days later by FACS. Results shown are from one experiment, and equivalent results were obtained by Dr Melvyn Yap. Error bars show standard error.

The data in figure 5.4 show that saturating cells at MOI of over 10 with viral particles before challenge with a second dose of GFP virus reduces titres on both HeLa CD4 and NP2* cells by 20-25% virus when the envelope protein is VSV-G, but has no clear effect when the envelope protein is MCR. Titres of MCR pseudotyped virions on NP2* cells did not change significantly, with a small increase of 6.6%, as might be expected for restriction factor negative cells. Titres on HeLa CD4 cells were well below 1%, that is, below the reliable analytical detection level of FACS, even after pre-challenge with saturating levels of MCR Env-pseudotyped viral particles. This makes any assessment of fold-change in titres difficult, and would strongly suggest that the nature of the block to infection in HeLa CD4 cells is such that it is not a saturable factor, or that any effect is insignificant. Abrogation of $Fv1^n$, $Fv1^b$ or Trim5α, on the other hand, increases titres of challenge virus by approximately 50-,

10- and 50-fold respectively (Dodding et al. 2005). Thus it must be concluded that Lv2 restriction is not caused by a saturable factor.

The existence of a dominant, saturable factor that was ultimately found to be Trim5α was also strongly suggested by experiments in which challenge of heterokaryons made from fusion of Refl-negative and Refl-positive cells gave titres equivalent to those seen in Refl-positive cells alone (Cowan et al. 2002). Schmitz et al. reported that attempts to assess the dominance of Lv2 restriction by creation of heterokaryons were unsuccessful and uninterpretable, as Lv2 restriction was not evident in heterokaryons, but was also quashed in homokaryons created from fusion of Lv2-restrictive cells (Schmitz et al. 2004). This would suggest that either plasma membrane disturbances or intracellular rearrangements from this process were responsible for any Lv2 knockdown, rather than alteration of a restriction factor. It is therefore important to consider the stage of the infectious cycle at which MCR is blocked.

5.5 Production of strong stop DNA is reduced in HeLa CD4 cells challenged with MCR pseudotyped HIV

Schmitz et al. detailed experiments with quantitative PCR, designed to compare ratios of early transcripts obtained in HeLa CD4 and U87* cells, for both MCR and MCN viruses (Schmitz et al. 2004). Data shown were the ratio of copies in U87* cells compared to HeLa CD4 cells (U87*: HeLa CD4), for both MCR and MCN at 4 timepoints; 1, 2, 6 and 18 hours. After 1 hour, there were 8x as many strong stop transcripts in U87* cells compared to HeLa CD4 for both MCR and MCN. This means that there are 8x as many MCN strong stop transcripts in U87* cells as in HeLa CD4 cells, and 8x as many MCR strong stop transcripts in U87* cells compared to HeLa CD4 cells. The ratio is the same for both viral clones after 1 hour. After 2 hours, the same ratio of U87*: HeLa CD4 strong stop transcripts for MCN is 20x, and for MCR, 8x. Therefore, it would appear that after 2 hours replication of MCN is more dramatically inhibited than that of MCR in HeLa CD4 cells. At both the 6 and 18 hour timepoints, however, there is no difference in the U87*: HeLa CD4 ratio of transcripts for MCR and MCN. What is not commented on, however, is that for both MCR and MCN this ratio is 25. This means that there are 25x more transcripts seen

in U87* cells compared to HeLa CD4 cells. Considering this ratio for MCN, it is not entirely clear how the virus then continues to generate an equivalent titre on HeLa CD4 and U87* cells. The conclusion drawn from these data in the paper is that 'equivalent levels of transcripts were observed at the 6- and 18-h timepoints, therefore MCR and MCN represent prCBL-23 and CBL23 described previously in (McKnight et al. 2001)'. However, the reference in this statement to 'equivalent levels' refers exclusively to the proportions of MCN and MCR (i.e. the fact that at the timepoints of 6 and 18 hours, both MCR and MCN produced 25x more strong stop transcripts on U87* cells compared to HeLa CD4 so MCR and MCN were equivalent to each other). This does not seem to support a conclusion of unhindered entry of either MCR or MCN to HeLa CD4 cells as there are 25x less strong stop transcripts than in U87* cells.

It is not possible to directly compare ratios obtained in experiments described here to these data, as MCN appeared unviable. On consideration, however, an important conclusion to have drawn from the previous MCR data alone would have been the obvious one, that as there are between 8-25x less strong stop transcripts made in HCD4 cells compared to U87* cells and that as these are the earliest product of reverse transcription, it cannot be maintained that reverse transcription is occurring at equivalent levels in HeLa CD4 and U87* cells.

In consideration of how a pre-reverse transcription block would manifest itself in qPCR data, an important question is what sort of difference in levels of early RT transcripts would be expected in cells that were restricted pre-reverse transcription compared to cells that were either restricted after reverse transcription or fully permissive? Data are presented in references (Yap et al. 2007) and (Yap et al. 2006) that show levels of reverse transcription products in cells expressing Owl Monkey Trim5CypA or one of several artificial constructs, all of which block pre-reverse transcription, compared to cells which express no restriction factor. The difference in the levels of strong stop products between the two is between 6 and 9-fold. Taking this as a standard for the expected difference in levels between a pre-reverse transcription block and permissive cells, MCR is most definitely restricted before reverse transcription in HeLa CD4 cells compared to U87* cells, and by the same

token, MCN is moderately inhibited (~2-3x, (Marchant 2006) or heavily so (5-25x (Schmitz et al. 2004).

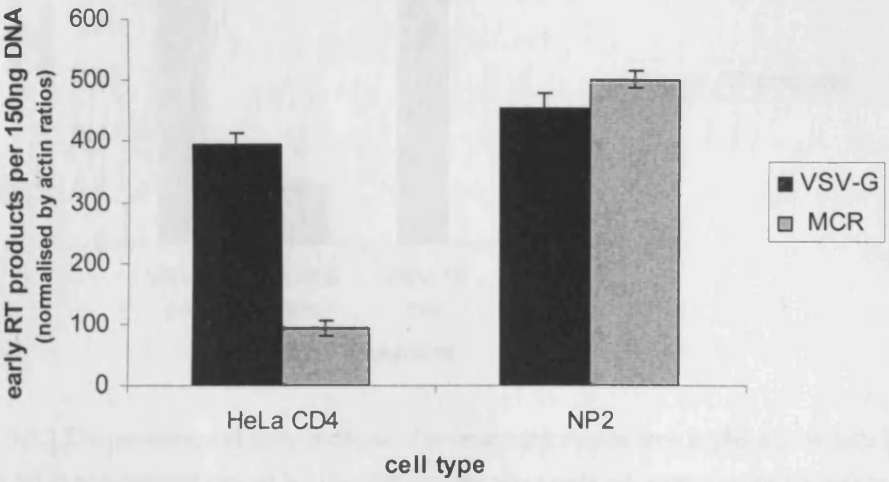


Fig. 5.5.1 Viruses pseudotyped with VSV-G, but not MCR, successfully reverse transcribe in HeLa CD4 cells. $2x10^5$ HeLa CD4 and NP2* cells were plated out. 24 hours later cells were overlaid with virions at MOI 0.8-1, and bound at 4°C for one hour. Infection was initiated by addition of medium and transfer to 37°C. After 6 hours cells were harvested, and DNA extracted using QIAamp DNA mini kit. The number of cells present was normalised by using ratios of actin for each sample run in parallel with early RT. Results shown are combined from 4 independent experiments, and error bars show sdm.

The data in figure 5.5.1 are the actual numbers of reverse transcription products obtained in HeLa CD4 and NP2* cells after infection with VSV-G or MCR pseudotyped virus. Levels of both virions produced equivalent levels of strong-stop transcripts in NP2* cells. In HeLa CD4 cells levels of strong stop products produced after MCR virus infection were 24% of those obtained from an infection with VSV-G pseudotyped virus.

A comparison was done for HeLa CD4 cells alone, for MCR and VSV-G pseudotyped virus at time points of 6 and 18 hours (still detecting strong stop transcripts) and the results shown in figure 5.5.2.

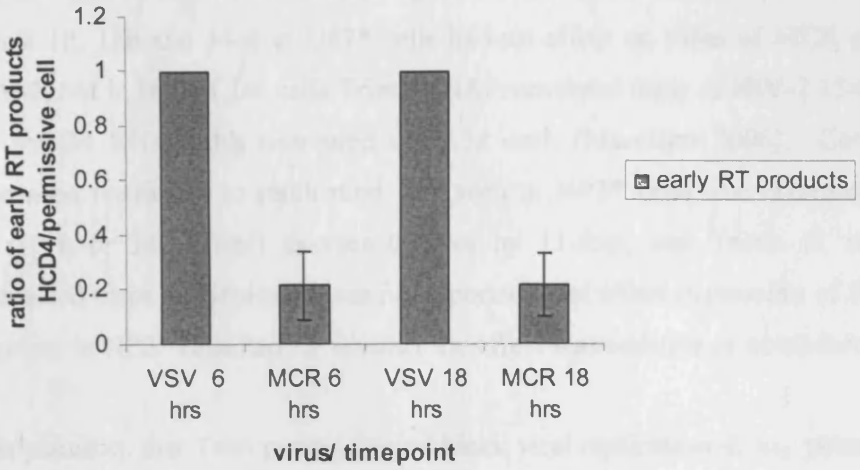


Fig. 5.5.2 The percentage of early products of reverse transcription seen in HeLa CD4 cells infected with MCR pseudotyped virions is 21% of those seen when cells are infected with VSV-G pseudotyped virions. This percentage does not increase over time. Data were processed from the experiments described in the legend to figure 5.5.1.

These data show that the appearance of appreciable levels of strong stop transcripts is not simply delayed in HeLaCD4 cells, but that it does not occur at all. Levels of MCR transcripts reach 21% of those of VSV-G (which is set to 100%) at both timepoints. From these data the former presumption that MCR pseudotyped virus is capable of efficient entry into HeLa CD4 cells must be questioned.

5.6 Introduction of Trims 1, 18 and 34 into NP2* cells does not create a block to infection

Human and rhesus macaque Trim5α are the causative agents of Ref1 and Lv1 respectively (Keckesova et al. 2004; Perron et al. 2004; Stremlau et al. 2004; Yap et al. 2004), and Trim1 has anti-N-MLV activity (Yap et al. 2004). As there are over 70 members of the Trim family of proteins, and many have as yet unidentified roles in the cell, there may be more with anti-viral activity (Yap et al. 2004; Nisole et al. 2005). It was suggested that Trim proteins may be responsible for Lv2, and a study done on members of this family that, like Trim5α, contain a SPRY domain (Marchant 2006), Trims 1, 6, 18 and 34.

It was reported that RNAi directed against Trims 1, 18 and 34 (specifically isoforms Trims 1β, 18β and 34α) in U87* cells had no effect on titres of MCR pseudotyped HIV-2, but in HeLa CD4 cells Trim1 RNAi recovered titres of HIV-2 15x, and Trims 18 and 34 RNAi both recovered titres 5x each (Marchant 2006). Conversely, an increased restriction to replication was seen in NP2* cells over-expressing Trim 1, Trim 18 or 34. Trim1 decreased titres by 11-fold, and Trims 18 and 34 each decreased titres by 6-fold. It was not reported what effect expression of all 3 proteins together in NP2* cells had, or whether the effect was additive or not (Marchant 2006).

It is plausible that Trim proteins could block viral replication at any point in the life-cycle, or even affect surface expression of CD4 or CXCR4, and this could explain data presented in 5.1-5.5 if these Trims were found to be expressed at elevated levels in HeLa CD4 cells compared to U87* or NP2* cells. Trims 1β, 18α and 34α (referred to in experiments discussing my data as 1, 18 and 34) were cloned into pLgatewayIRESYFP by Dr Melvyn Yap, and transduced into NP2* and HT1080 cells. The plasmid pLTva950IRESYFP was used as a control, to assess the effects of transduction on the cells, as expression of Tva950 should not affect titres of virions that do not use this receptor. 3 days later cells were challenged with either VSV-G or MCR pseudotyped NB-MLV and HIV.

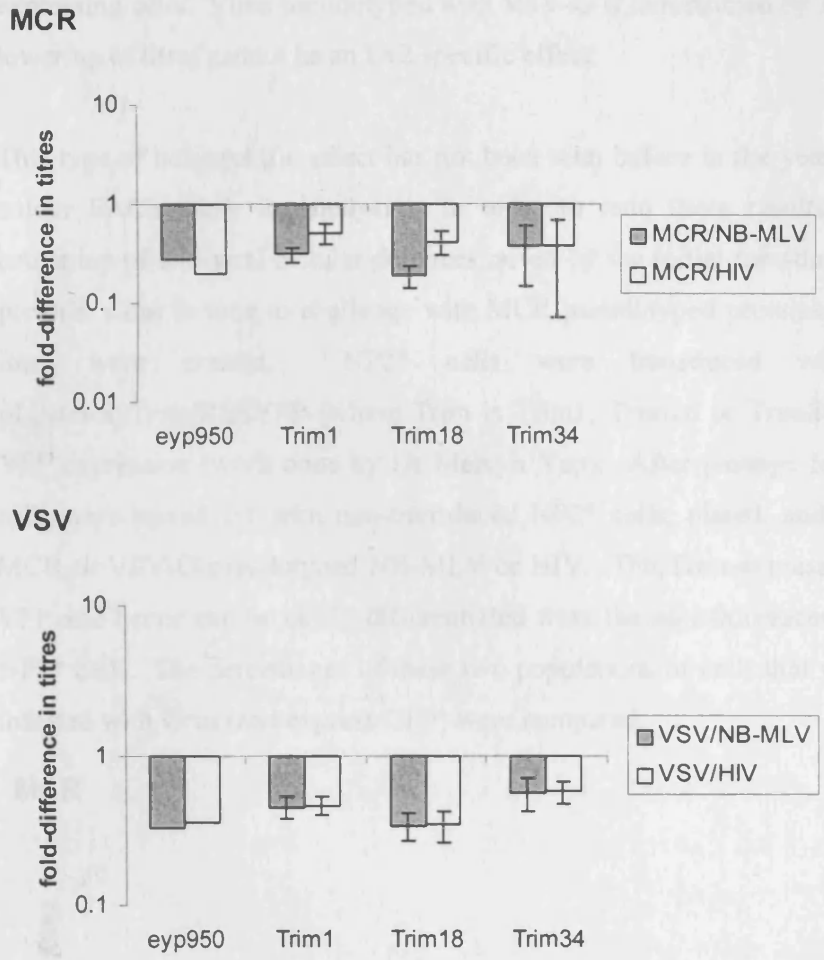
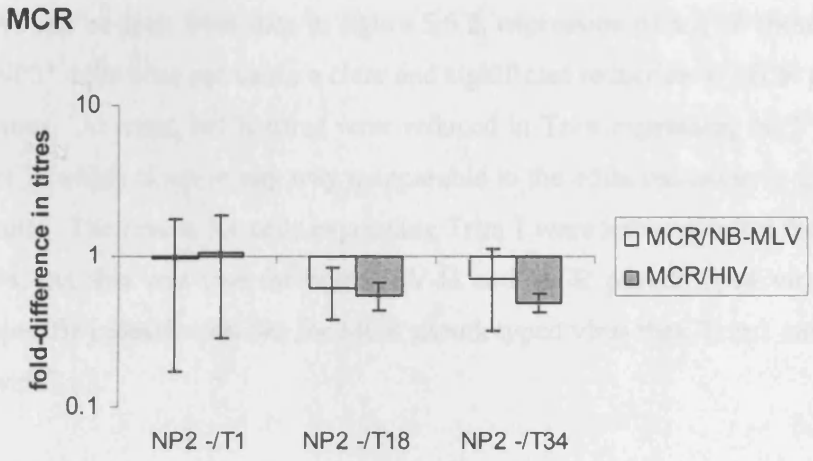


Fig 5.6.1 A non-specific reduction in VSV-G-pseudotyped viral titres is seen on addition of Trims 1, 18, 34, or Tva950 to NP2* cells. This reduction was seen whether the viral envelope was MCR (i.e. Lv2 sensitive) in the top graph, or VSV-G (i.e. Lv2-insensitive) in the bottom graph, and for both HIV-1 and NB-MLV cores. Fold-difference was calculated from the ratio of viral titres on cells expressing Trim or eyp950, compared to cells that were not expressing an extra factor. The results shown are combined from 3 separate experiments apart from eyp950, which was done twice.

As is clear from the size of the error bars in the MCR graph (particularly for HIV) the results varied considerably between experiments. Cells expressing eyp950 were included to ensure that there was no non-specific effect on titres from prior challenge with vector. However, as challenge with eyp950 vector effected a similar reduction in titres of MCR pseudotyped virus compared to any of the three Trim proteins, a non-specific effect seems to be what is shown rather than any specific block. Additionally, as shown in fig 5.6.1, VSV-G pseudotyped virus titres in Trim-expressing cells are similarly reduced to between 34 to 56% of those in non Trim-

expressing cells. Virus pseudotyped with VSV-G is unrestricted by Lv2 therefore this lowering of titres cannot be an Lv2 specific effect.

This type of non-specific effect has not been seen before in the years of using the 2-colour FACS assay for analysis. In order to redo these results, and avoid any activation of anti-viral cellular defences raised by the initial transduction of the Trim proteins close in time to challenge with MCR pseudotyped proteins, three stable cell lines were created. NP2* cells were transduced with the vector pLgatewayTrimIRESYFP (where Trim is Trim1, Trim18 or Trim34) and sorted for YFP expression (work done by Dr Melvyn Yap). After passage for several weeks, cells were mixed 1:1 with non-transduced NP2* cells, plated, and challenged with MCR or VSV-G pseudotyped NB-MLV or HIV. The Trim-expressing cells express YFP and hence can be easily differentiated from the non-fluorescent, Trim-negative NP2* cells. The percentages of these two populations of cells that were successfully infected with virus (and express GFP) were compared.



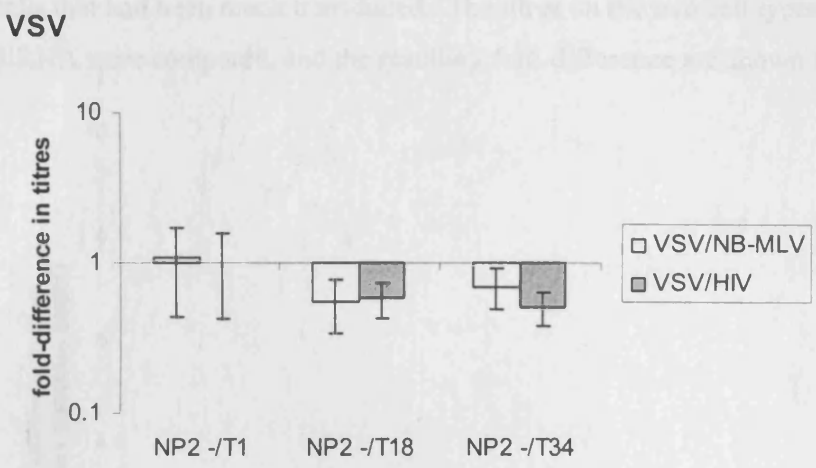


Fig 5.6.2 Long-term expression of Trims 1, 18 or 34 does not lead to a consistent and significant decrease in virion infectivity on NP2* cells. NP2* cells were transduced with trims 1, 18 and 34, sorted for YFP expression, and passaged for several weeks. They were mixed 1:1 with the parental NP2* cells and challenged with VSV or MCR pseudotyped NB-MLV or HIV-1. Shown are the combined results of two independent experiments. Error bars represent sdm.

As can be seen from data in figure 5.6.2, expression of any of these Trim proteins in NP2* cells does not cause a clear and significant reduction in MCR pseudotyped virus titres. At most, MCR titres were reduced in Trim-expressing NP2* cells by a factor of 2, which is not in any way comparable to the >50x reduction in titres in HeLa CD4 cells. The results for cells expressing Trim 1 were less consistent than for Trims 18 or 34, but this was true for both VSV-G and MCR pseudotyped virus. If there is no specific reduction shown for MCR pseudotyped virus then Trim1 cannot be connected with Lv2.

5.7 Expression of anti-Trim1 SiRNA does not diminish the block to infection in HeLa CD4 cells

As Trim1β had been previously reported to cause the highest drop in titres when expressed in NP2* cells compared to Trims 18 and 34 (Marchant 2006), and the results seen here had been somewhat varied, it was important to ensure that these were reliable, preferably by another method. SiRNA directed against Trim1 was designed and verified by Dr Melvyn Yap and transduced into HeLa CD4 cells. Three days later cells were challenged with MCR pseudotyped HIV and titres compared to

cells that had been mock transduced. The titres on the two cell types with and without SiRNA were compared, and the resulting fold-difference are shown in figure 5.7.

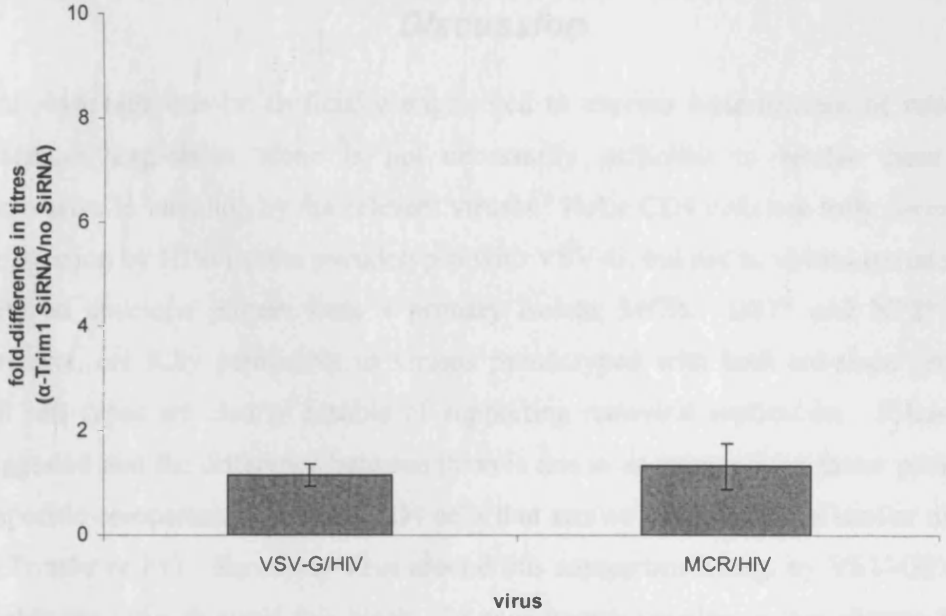


Fig. 5.7 Expression of anti-Trim1 SiRNA in HeLa CD4 cells does not affect titres of VSV-G or MCR pseudotyped HIV-1. HeLa CD4 cells were transduced with anti-Trim1/YFP vector, and then challenged with VSV-G and MCR pseudotyped HIV-1. The fold-difference in titres between cells expressing the SiRNA and null cells was calculated, and is shown above. A fold-difference of 1 indicates that the SiRNA has no effect, and >1 that the SiRNA has increased titres. Shown are the combined results from two experiments. Error bars represent the variation in ratios from several volumes used within the experiments.

Titres of both the control VSV-G and the MCR pseudotyped HIV are indistinguishable across the two sets of HeLa CD4 cells, regardless of the SiRNA against Trim1 in one set. These data indicate that Trim1 does not play a role in reducing titres of MCR pseudotyped virus in HeLa CD4 cells. This, and the previous experiment, reinforces the likelihood that Trim proteins 1, 18 and 34 are not the causative agents for the Lv2 phenotype but that different factors or mechanisms are responsible.

If this is the case, then the question now arises as to when the block to infection actually occurs, and what could possibly be the cause. This was investigated in experiments reported in chapter 6.

Discussion

Although cells can be artificially engineered to express combinations of retroviral receptors, expression alone is not necessarily sufficient to render these cells permissive to infection by the relevant viruses. HeLa CD4 cells are fully permissive to infection by HIV-1 cores pseudotyped with VSV-G, but not to virions pseudotyped with an envelope protein from a primary isolate, MCR. U87* and NP2* cells, however, are fully permissive to viruses pseudotyped with both envelope proteins. All cell types are clearly capable of supporting retroviral replication. It has been suggested that the difference between them is due to an intracellular factor present in a specific compartment of HeLa CD4 cells that acts on viral cores in a similar manner to Trim5α or Fv1. Rerouting virus around this compartment (e.g. by VSV-G) would enable the virus to avoid this block. In experiments detailed in this chapter I have studied the phenomenon carefully by standard and new techniques, and come to the conclusion that while there is a block to infection in HeLa CD4 cells, this is unlikely to be caused by a Fv1- or Trim5α-like restriction factor.

It should be noted that experiments that compare VSV-G and MCR are at best relative, as two different virus populations must be used with different efficiencies of production and binding. Experiments with MCN were completely unsuccessful, as it appeared to be totally non-functional on all cell types; viruses pseudotyped with this envelope failed to enter any cells. An attempt to recapitulate the Lv2 phenomenon using Tva800 and Tva950 in HeLa CD4 cells was also unsuccessful, as no difference in viral replication was seen with either receptor. Thus all experiments described here were done with VSV-G and MCR envelopes.

A characteristic of restriction by Fv1 and Trim5α is that the block to infection is saturable. This is highly indicative that a specific factor is causing the block to infection in an infectious cycle that could otherwise be productive. As the block in HeLa CD4 cells cannot be overcome with high titres of viruses, it is hard to conclude that it must be caused by a specific factor, as there is no indication that the infection

would otherwise be successful. The use of the envelope protein VSV-G merely confirms that the cell is able to support viral replication. It does not prove or disprove the existence of a cell-compartment specific inhibitory factor.

The detection of significant levels of reverse transcription intermediates, while not infallible, is an indication that entry is occurring at levels relevant to a successful infection. Previous experiments that investigated levels of products of reverse transcription showed either that they simply could be detected (McKnight et al. 2001), or compared levels obtained with MCN and MCR-pseudotyped virions (Schmitz et al. 2004). In the former, levels of reverse transcripts were not equivalent in U87*, HeLa CD4 and HOS cells. Results were displayed as a PCR gel analysis of LTR-*gag* from between 100 and 10,000 cells. Levels of transcripts were approximately equivalent for 100 U87* and 10,000 HeLa CD4 cells, i.e. they were 100x lower in HeLa CD4 cells. Levels in HOS cells were somewhat higher, and levels on the lane obtained from 3,333 HOS cells were equivalent to 333 U87* cells. It was not discussed whether reverse transcription occurring pre-infection (i.e. within the virions) or in endosomes before lysosomal destruction could be sufficient in the larger samples of HeLa CD4 cells to produce a false positive, but this could indeed be the case (Trono 1992; Zhang *et al.* 1993). In the (Schmitz *et al.* 2004) reference, levels of reverse transcription intermediates were equivalent for MCN and MCR, as discussed in section 5.5. These results were presented as a ratio of reverse transcription quantities obtained on U87* and HeLa CD4 cells for the two envelopes. This ratio varied between 8-fold at 1-2 hours, to 25-fold at 6-18 hours. Therefore while levels of reverse transcription intermediates may be equivalent between infections mediated by the MCR and MCN envelopes, levels between the two cell types are not. That is, there are between 8 to 25-fold fewer products of reverse transcription being synthesised on HeLa CD4 cells compared to U87* cells. The pre-reverse transcription block seen in Trim5Cyp-positive cells with susceptible virus leads to a drop in reverse transcription intermediates of at least 5-fold, so an 8 to 25-fold block is commensurate with a lack of significant synthesis of reverse transcription products (Yap *et al.* 2006). This conclusion, then, precludes the hypothesis of a factor as responsible for a specifically post reverse transcription block to infection in HeLa CD4 cells.

This means that the phenomenon described as Lv2 must be reconsidered. As originally characterised, this was described as a post-entry lentiviral restriction with components of the restriction attributable to both Gag and Env, that was due to the targeting of the virus into an inappropriate sub-cellular compartment (Schmitz *et al.* 2004). Pseudotyping with VSV-G overcame the Env-dependent component of the restriction, and also removed the Gag-dependency. Experiments described here confirm the original premise on which Lv2 is based, namely that some cells can be infected by MCR pseudotyped virus, and that other cells from the same species expressing the same combination of receptor and co-receptor cannot. As described, the block does not fall under the current understanding of a restriction factor (Goff 2004; Sorin and Kalpana 2006). However, this would simply mean that the definition of what constitutes a restriction factor would have to be extended if it could be proven conclusively after an elimination of all other possibilities in a sort of molecular biology version of Occam's razor, that the block to infection could not be attributable to any other factors. The conclusion from a re-interpretation of results from previous studies and those described here does not suggest that this elimination has been fully carried out, and cannot support a hypothesis that a restriction factor is definitely responsible for the block to infection. The name Lv2, standing as it does for lentivirus restriction factor 2, is probably a misnomer. The block is not saturable, affects other CD4/CXCR4-tropic envelopes and does not permit entry of virions into the cell at levels commensurate with a successful infection. These issues do not point towards a restriction factor being responsible, and indeed do not rule out other irregularities or differences in cell biology between NP2*, U87* and HeLa CD4 cells as being primarily responsible. This implies that the reason why HeLa CD4 cells are refractory to infection by MCR pseudotyped virions must be sought elsewhere.

Chapter 6

The Block to Infection in HeLa CD4 Cells is at Entry

It was shown in chapter 5 that viral cores pseudotyped with MCR are able to infect NP2* cells, but not HeLa CD4 cells. This block to infection was previously attributed to an intracellular restriction factor similar to Fv1 and Trim5α but with a route of entry component. Experiments described in chapter 5 show that conclusive evidence of reverse transcription, and therefore entry of the virus into the cell was not found, leading to the conclusion that the block may not be due to a restriction factor at all. The primary cause of the block to replication probably lies in some aspect of the differences in cell biology between HeLa CD4, U87* and NP2* cells. This being the case, further experiments were carried out to try and ascertain the nature of this block, how it prevents the virus from infecting the cell, and at what point in the infectious cycle it does this. These experiments are described in this chapter.

Results

6.1 CD4 and CXCR4 are expressed on HeLa CD4 cells

In order to ensure that the cells I was using expressed sufficient amounts of the necessary receptor and co-receptor combination for entry using MCR (McKnight et al. 1998), confluent flasks of HeLa CD4, U87* and NP2* cells were harvested, lysed and sampled by Western blot for CD4 and CXCR4 expression. HOS CD4/CXCR4 (HOS) cells were obtained from the AIDS Reagent program (NIBSC, Potters Bar, UK) and were used to provide a positive control for CXCR4 expression. *Mus dunni* cells were also assayed as a negative control for both proteins. Input protein level was checked by probing for GAPDH.

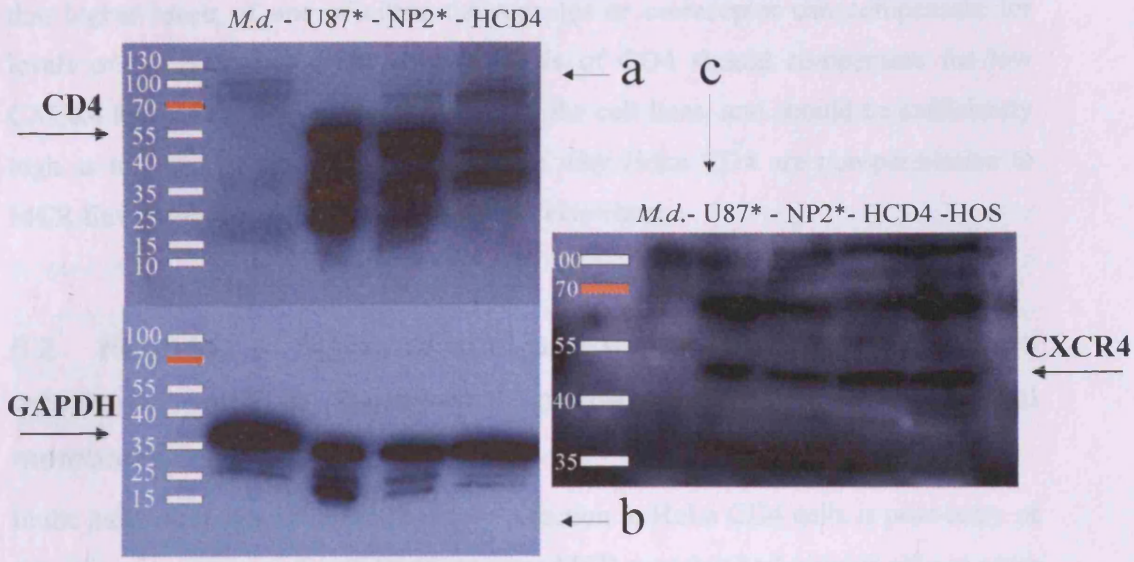


Fig. 6.1 Western blot of total levels of CD4 and CXCR4 in cellular lysates. Samples were probed for a) CD4, b) GAPDH and c) CXCR4. The same blot in a) was stripped and reprobed for b).

The Western blot shows that although CD4 is artificially expressed in all cell lines, levels do differ between cells. The levels appear to be highest in U87* cells, which are permissive to MCR Env, but the lower levels seen in HeLa CD4 cells are still readily detectable. HeLa CD4 cells express CXCR4 endogenously and the parental HeLa cell line is suggested as a positive control for CXCR4 expression in published protocols for several CXCR4 antibodies. From this Western blot, total levels of CXCR4 across the 4 cell lines appear to be broadly similar. Specific primary antibodies that readily bind to CXCR4 are not available, and when blots are incubated at 4˚C overnight some non-specific binding occurs, as the bands at around 100kDa (seen in *Mus dunni* cells) and possibly 70kDa show. So, total levels of CXCR4 are similar across U87*, NP2* and HeLa CD4 cells, but levels of CD4 vary, although there is no guarantee that expression of either CD4 or CXCR4 means that the protein is being properly processed and trafficked in a particular cells line.

There is evidence that levels of CD4 and CCR5 necessary for entry must be over a threshold level and that levels of CCR5 can be much lower if CD4 levels are high (Platt et al. 1998). It could be argued that part of the difference in titres on these two cell types compared to HeLa CD4 cells may relate to the elevated level of artificial CXCR4 over-expression in U87* and NP2* cells compared to HeLa CD4 cells, in which CXCR4 is produced endogenously. If, however, as is true for CD4 and CCR5

that higher levels of one of either the receptor or co-receptor can compensate for levels of the other being low, higher levels of CD4 should compensate for low CXCR4 levels. CD4 is overexpressed in all the cell lines, and should be sufficiently high as to not limit binding, and the reason why HeLa CD4 are non-permissive to MCR Env pseudotyped virus must be sought elsewhere.

6.2 Progress of fusion of viral and cellular membranes can be monitored with a fluorescent protein targeted to the viral membrane

In the judgement of whether the block to infection in HeLa CD4 cells is post-entry or not, it is of key importance to know whether MCR pseudotyped virus is able to enter cells. In order to ascertain this, a different method of assessing entry to PCR was called for. In 2007 it was reported that red membrane-labelled virions are produced in cells that express a fluorescent fusion protein comprised of mCherry and the 15 N-terminal amino acids of Src (s15-mC) (Campbell *et al.* 2007b). These 15 amino acids target mCherry to the plasma membrane of the producer cell, and the viral particles are coated with red membrane as they leave the cell (Rodgers 2002; Campbell *et al.* 2007b). The coating is maintained as the viral envelope binds to its receptor on a target cell, and disperses after fusion between the viral and cellular membranes has taken place. If the virus is endocytosed but fusion out of the endosomes is prevented (for example when fusion of VSV-G envelope is blocked by bafilomycin A1) then the red signal remains intact (Campbell *et al.* 2007b). This system was chosen to aid analysis of whether fusion was successfully taking place between MCR pseudotyped viruses and HeLa CD4 cells. If the proportion of virions that lost their red coating after binding remained unchanged between NP2*, U87* and HeLa CD4 cells then, this would indicate that fusion was occurring at equivalent levels in the different cells, and the blockage to replication in HeLa CD4 cells was further downstream in the infectious pathway after entry.

In order to monitor viral particles after the fusion event, they needed to be dually labelled with GFP-vpr, which labels the viral cores, as well as with the S15-mC dye (McDonald et al. 2002). Viral particles outside the cell, and those bound to cells pre-fusion would be both green and red; those that had successfully completed a fusion

event would be green only. Counting the proportions of each should reveal in which cells fusion was blocked. Repeated washing was carried out pre-incubation and pre-fixation to ensure that only virions tightly bound to receptor and not free virions comprised the green and red populations present. In order to ensure that as few virions as possible progressed beyond dispersal of the GFP-vpr signal and hence beyond detection, infections were initially carried out for half an hour. However, this short time frame did not allow a sufficient number of virions to fuse to differentiate between those that were inhibited, and those that were not, so an infection period of 2 hours was eventually used.

293T cells were initially transfected with 5 plasmids (Gag-pol, Env, package (CSTKW), GFP-vpr and s15-mC) at equal ratios but this did not result in any virus being produced, as high levels of s15-mC seem to have a negative impact on viral viability. Varying the ratio of s15-mC to the other plasmids (from 1:1 to 1:10) resulted in viruses that were increasingly infectious as the proportion of s15-mC decreased, as shown in figure 6.2.1.

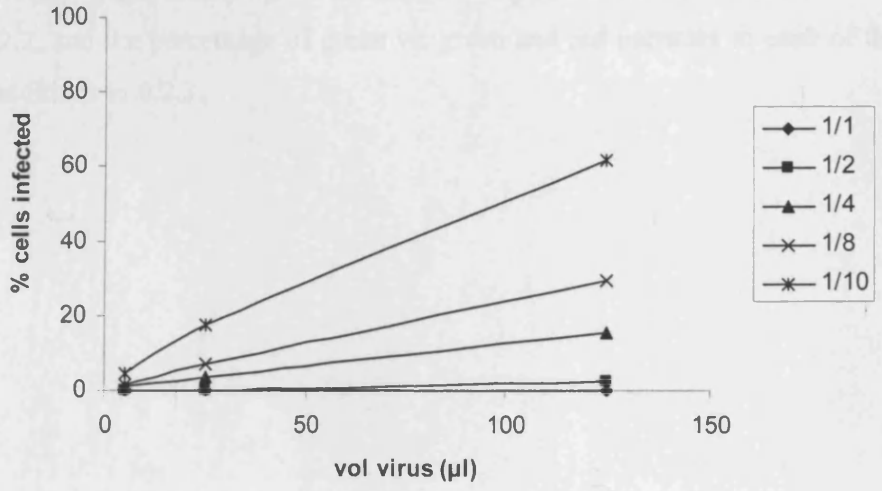


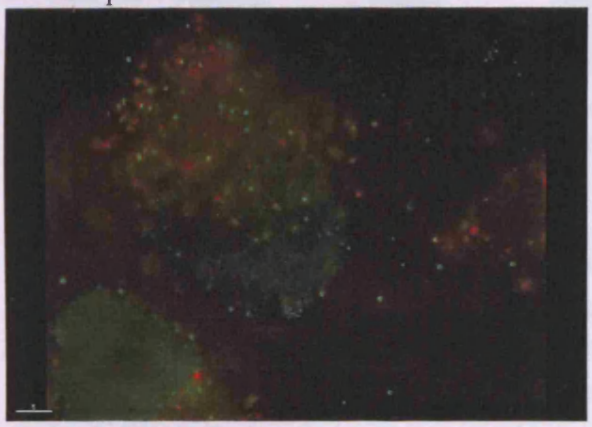
Fig. 6.2.1 Reducing the ratio of s15-mC:CSGW increases the infectivity of virions produced. 293T cells were transfected with gag-pol, VSV-G, CSGW, GFP-vpr and s15-mC at ratios varying between 1:1:1:1:1 to 1:1:1:1:10, as shown in the key to the graph above. 5-125µl virus was titrated on $5x10^4$ dunni cells, and the results analysed by FACS (for GFP expression) three days later. Shown are the results of one experiment, typical of 3 carried out.

In order to ensure that none of the nascent viral particles should escape being labelled red so that particles would be erroneously considered to have undergone fusion, it was deemed desirable to keep the concentration of s15-mC as high as possible. Because of this, although a further decrease in the ratio of s15-mC to CSGW to 1:20 yielded particles with slightly higher infectivities compared to a 1:10 ratio, the ratio of 1:10 was decided upon for further experiments.

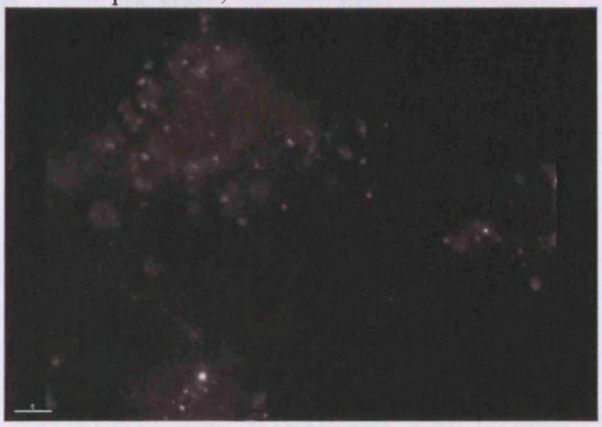
In order to gauge the utility of this method for assessing whether fusion had been completed or not, green and red viral particles were pseudotyped with VSV-G envelope protein, bound to HeLa CD4 or U87* cells at 15°C for two hours, and then incubated at 37°C for two hours, all steps in the presence or absence of NH_4Cl. NH_4Cl is well documented to prevent initiation of fusion by the VSV-G envelope (Aiken 1997), and the virus remains trapped in endosomes. After infections, cells were fixed and stained with DAPI, and at least 10 pictures were taken using a 100x lens at discrete points on the slide. Files were randomised by a colleague before the pictures were opened in Adobe photoshop, and scored blind for overlapping red and green, or green alone, signals. Examples of pictures used to obtain data are shown in 6.2.2, and the percentage of green vs. green and red particles in each of the different conditions in 6.2.3.

a. Viral particles.

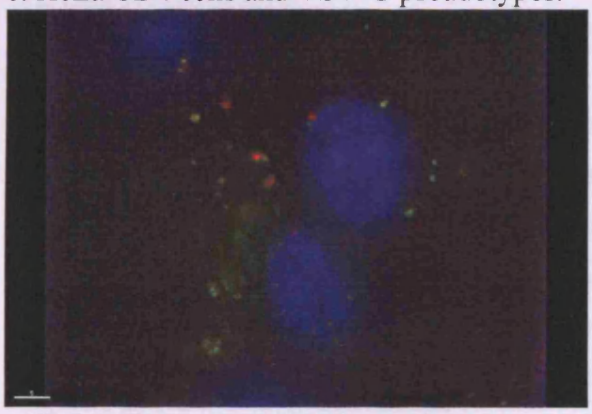
b. Viral particles, red channel.



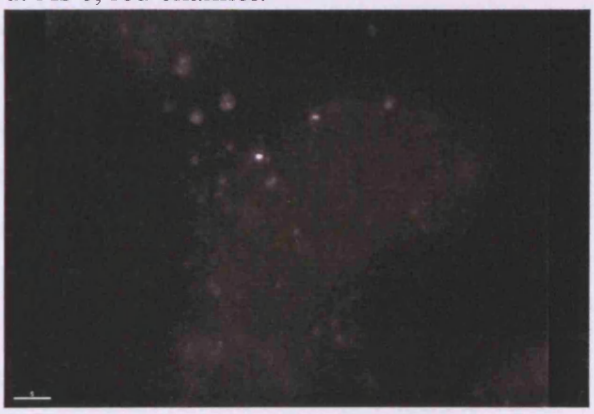
c. HeLa CD4 cells and VSV-G pseudotypes.



d. As c, red channel.



e. HeLa CD4 cells, VSV-G pseudotypes incubated with NH_4Cl.



f. As e, red channel.

Fig. 6.2.2 Viral particles can be localised with GFP-vpr, and their membranes stained with S15-mCherry. These particles permit assessment of fusion with cellular membranes. Nuclei are stained in blue (where shown) with DAPI, viral particles (fused) are shown by green, and viral membranes red in the left-hand pictures. In pictures on the right-hand side, the blue and green channels are removed and the red signal only is shown, in black and white for clarity. a) and b) show the viral supernatant only. All green particles also had a corresponding red signal, so were deemed suitable for use in assessment of fusion. c-f) Cells were spinoculated with virus at MOI >5 for 2 hours at 15°C, then incubated at 37°C for 2 hours before fixation with paraformaldehyde. Scoring of fusion was done blind by comparison of the location of the green and red signals for a minimum of 10 pictures per cell type per condition after pictures were randomised by a colleague. Shown are HeLa CD4 cells, but similar pictures were obtained and scored for U87* cells as well.

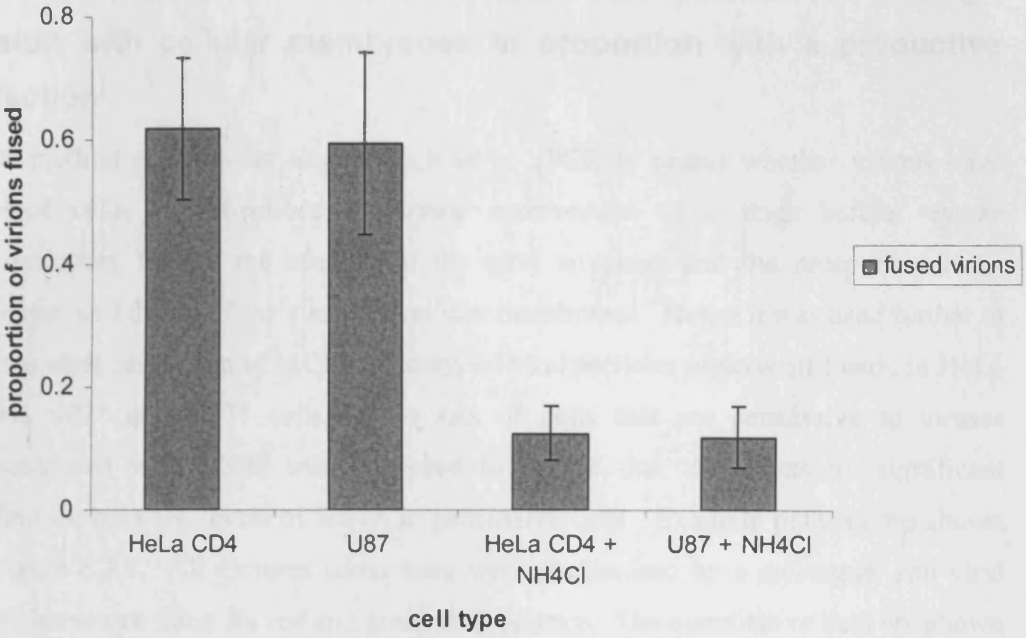


Fig. 6.2.3 Virions pseudotyped with VSV-G retain their viral (cherry red) membrane when NH_4Cl blocks fusion. Shown are the proportions of virions that have successfully fused with the cell, that is, they have lost their red membrane signal and evince GFP fluorescence only. The proportion of virions fusing in the presence and absence of NH_4Cl is the same for HeLa CD4 and U87* cells. The results shown are from 10 separate pictures taken per condition (examples are shown in figure 6.2.2), with over 500 virions per condition counted blind and scored for red or green fluorescence.

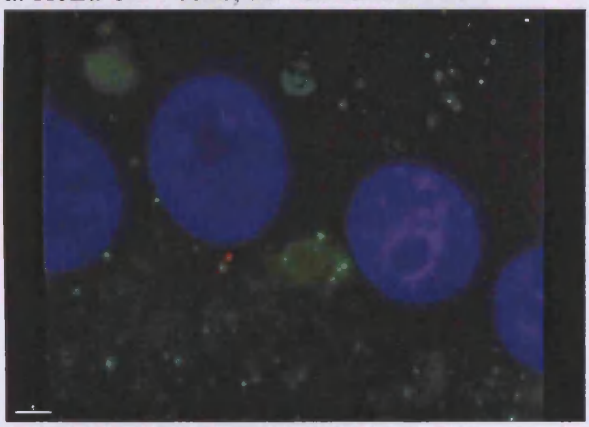
The data in figure 6.2.3 show that after 2 hours of incubation with media alone, 62 and 59% of viral particles had undergone fusion in HeLa CD4 and U87* cells respectively. These were the particles that were only fluorescent green, having lost their red membranes. If NH_4Cl was present, which blocks entry by VSV-G, successful fusion events were seen in only 12% of particles tallied, for both HeLa CD4 and U87* cells. As it is much harder to detect reliably the absence of a red label than its presence, it may be that the percentage of fusion events in cell is slightly lower than these percentages. However, even with that caveat there is still a clear and significant difference between the percentages of VSV-G viral particles that have lost their red virion membrane label in the presence or absence of NH_4Cl.

6.3 Membranes of HIV pseudotyped with MCR do not undergo fusion with cellular membranes in proportion with a productive infection

This method provides an alternative way to qPCR to assess whether virions have entered cells. Furthermore it permits assessement of a stage before reverse transcription, that of the binding of the viral envelope and the receptor and co-receptor, and fusion of the viral and cellular membranes. Hence it was used further to assess what proportion of MCR pseudotyped viral particles underwent fusion in HeLa CD4, NP2* and U87* cells. Two sets of cells that are permissive to viruses pseudotyped with MCR were assessed to ensure that there was no significant difference between levels of fusion in permissive cells. Example pictures are shown in figure 6.3.1. All pictures taken were were randomised by a colleague, and viral particles scored blind for red and green fluorescence. The quantitative data are shown in figure 6.3.2.

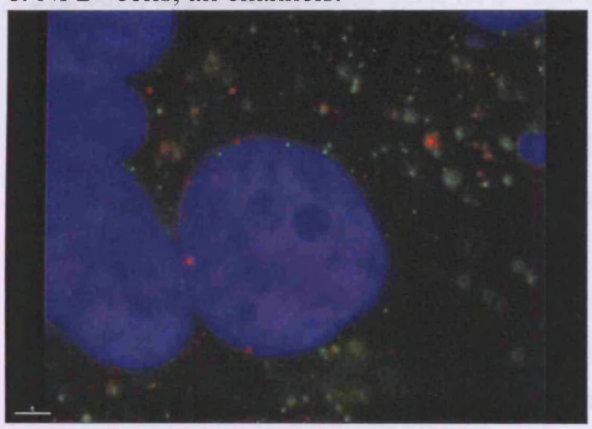
a. HeLa CD4 cells, all channels.

b. HeLa CD4 cells, red channel only.

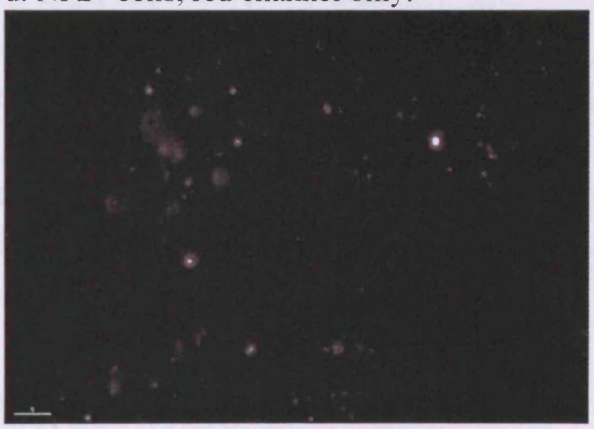


c. NP2* cells, all channels.

d. NP2* cells, red channel only.



e. U87* cells, all channels.



f. U87* cells, red channel only.

Fig. 6.3.1 MCR pseudotyped viral particles do not initiate successful fusion with the plasma membrane of HeLa CD4 cells. Particles applied to U87* or NP2* cells show fusion rates of over 60%, within the 2 hour time-frame. For HeLa CD4 cells the proportion fused was 15% of particles. Samples were prepared and assessed as described in the legend to fig 6.2.2. All pictures show MCR pseudotyped virions with different cell lines: a and b, HeLa CD4 cells; c and d, NP2* cells; e and f, U87* cells.

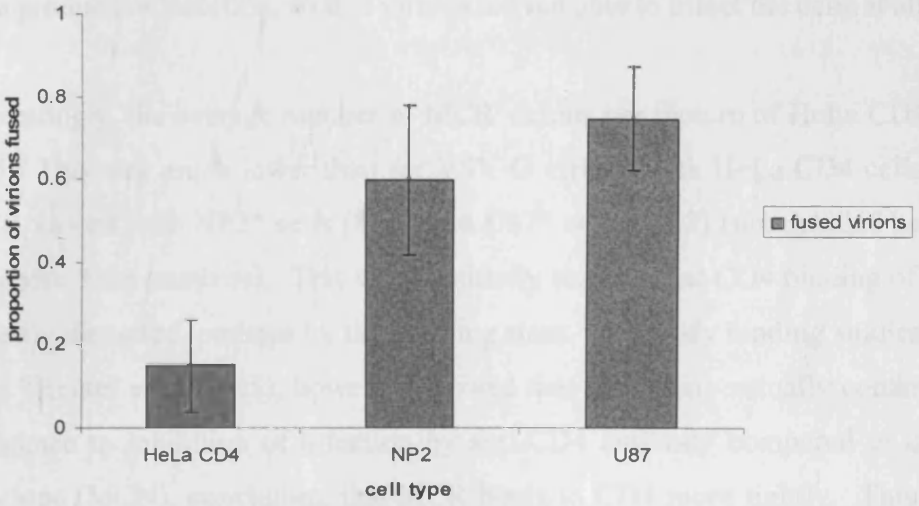


Fig. 6.3.2 A comparison of the proportion of virions that have successfully fused in HeLa CD4, NP2* and U87* cells when the viral envelope protein is MCR. Shown are the proportions of virions that have lost their cherry red label. Data encompass the results from at least 10 pictures taken per cell type, and scored blind for red and green fluorescence.

Once bound and visualised, the proportion of MCR virions that underwent fusion was 15%, 60%, and 75% for HeLa CD4, NP2* and U87* cells, respectively. Student's *t*-test was used to determine whether the difference between the number of fused virions was significant in pairs of pictures: NP2* and U87* cells for MCR; HeLa CD4 and either NP2* or U87* cells for MCR; HeLa CD4 cells with MCR or VSV-G pseudotyped virions when the latter were held under an NH_4Cl-block to fusion. The *t*-values obtained suggested that the difference between fusion levels in NP2* and U87* cells with MCR pseudotyped virus was not significant. The difference between fusion levels in either HeLa CD4 and U87* cells, or HeLa CD4 and NP2* cells was significant (see appendix 3 for numbers). Furthermore, the difference in fusion levels between HeLa CD4 cells with MCR pseudotyped virus, or HeLa CD4 cells with VSV-G pseudotyped virus held under an NH_4Cl-induced block to infection was not significant, i.e. there was effectively no difference in the proportion of virions fused.

This means that the level of fusion in HeLa CD4 cells incubated with MCR virus is commensurate with that of VSV-G virions under an NH_4Cl-induced blockage. This similarity strongly suggests that fusion of MCR pseudotyped virions with HeLa CD4

cells is prohibitively blocked, and entry is not happening to levels significant enough for a productive infection, so that virions are not able to infect the cells at all.

Interestingly, the average number of MCR virions per picture of HeLa CD4 cells was 21.5. This was much lower than for VSV-G virions with HeLa CD4 cells (93.2), or MCR virions with NP2* cells (81.7) and U87* cells (46.2) (not detailed here, but see appendix 3 for numbers). This would initially suggest that CD4 binding of MCR Env is easily disrupted, perhaps by the washing steps. Antibody binding studies by Reuter et al. (Reuter et al. 2005), however, showed that MCR Env actually conferred a 10x-resistance to inhibition of infection by anti-CD4 antibody compared to unrestricted envelope (MCN), concluding that MCR binds to CD4 more tightly. This difference was pinpointed to a glutamic acid residue at position 74 in MCR, compared to a glycine in MCN (Reuter et al. 2005). So it is more likely that MCR does not readily bind in the first place. As CD4 expression is foreign to all of the three cell types studied here, it is not immediately apparent why there should be such a difference in initial binding, whether it is due to initial expression and processing of the protein, or expression at the cell surface, or differences in cell type that become apparent after virus binding.

6.4 Inhibition of endocytosis or acidification of endosomes causes a small increase in replication of MCR NB-MLV in HeLa CD4 cells

After MCR binds to CD4 and CXCR4 at the cell surface, during a productive infection pH-independent fusion will occur without endocytosis. Endocytosis is not a productive route of entry into the majority of cells for virions pseudotyped with HIV envelopes (Pelchen-Matthews *et al.* 1995; Fredericksen *et al.* 2002). This is in direct contrast to the ASLV-A envelope and Tva800 and Tva950 receptors used in experiments discussed in chapters 3 and 4, which require entry into the endocytic pathway and a drop in pH to trigger fusion. The fact that virions pseudotyped with MCR are not able to infect HeLa CD4 cells means that the question must be asked as to whether instead of subsequent fusion and entry into the cytosol, endocytosis is instead occurring, the virus is unable to exit the endocytic pathway, and ends up degraded in the lysosomes.

This question was addressed by two previous studies into entry mediated by MCR Env (Marchant *et al.* 2005; Reuter *et al.* 2005). In one experiment, a concentration of 10mM NH_4Cl (which raises the pH inside lysosomes) was sufficient to raise titres on restrictive Ghost/X4 cells by 10x, while increasing titres on U87* cells by only 2x (Reuter *et al.* 2005). In a second study, addition of NH_4Cl decreased the difference in titres of MCR pseudotyped virus between HeLa CD4 cells and U87* cells from 30- to 5-fold, and hypertonic sucrose (which inhibits endocytosis from the cell surface) reduced restriction from 40-fold to 5-fold (Daukas and Zigmond 1985; Heuser and Anderson 1989; Marchant *et al.* 2005). However, addition of bafilomycin A1, an inhibitor of the H^+-ATPase, did not increase titres on HeLa CD4 cells. Both NH_4Cl and sucrose were purported to increase the level of virus infecting HeLa CD4 cells, rather than to reduce the levels successfully infecting U87* cells. These results suggested to the authors of the second study that "Lv2 occurs along a pH-dependent route" (Marchant et al. 2005).

In an effort to address possible differences in cell biology between cell types, a study of the basal rate of uptake of CD4 by endocytosis in restrictive Ghost/X4 and U87* cells was carried out (Reuter *et al.* 2005). It was found to be similar in the two cell types, with 48% and 53% of CD4 molecules remaining at the cell surface after 40 minutes for Ghost/X4 and U87* cells respectively. Previously, a separate study had found the rate of recycling of CD4 to be 1.5-2% per minute in HeLa CD4 cells which is potentially somewhat higher (Pelchen-Matthews et al. 1989), equating to 60-80% over 40 minutes. Neither study took account of any alteration (i.e. stimulation) in endocytic rate that would be occasioned by viral binding.

In order to assess how these data could fit together, infections were carried out of HeLa CD4 cells by MCR pseudotyped virus in the presence of the endocytic inhibitors NH_4Cl and sucrose. Control experiments with VSV-G pseudotyped virus in HeLa CD4 and NP2* cells were also carried out.

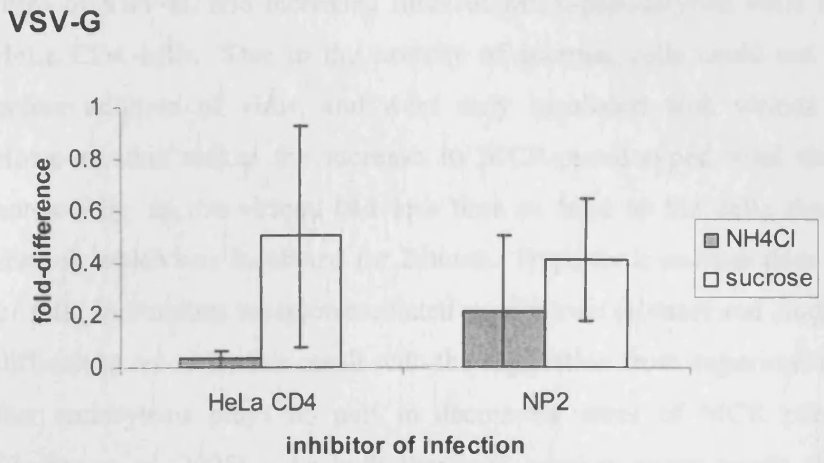


Fig. 6.4.1 Inhibition of endosomal acidification or endocytosis reduces titres of VSV-pseudotyped virus. HeLa CD4 and NP2* cells were plated and infected at MOI 0.5 in DMEM, DMEM + 40mM NH_4Cl or DMEM + 0.45M sucrose. Cells were incubated with virus for 2 hours, before the replacement of medium with DMEM only. Cells incubated with sucrose received fresh medium after 40mins, due to toxic effects of sucrose. Results were analysed by FACS, and shown are combined from two experiments.

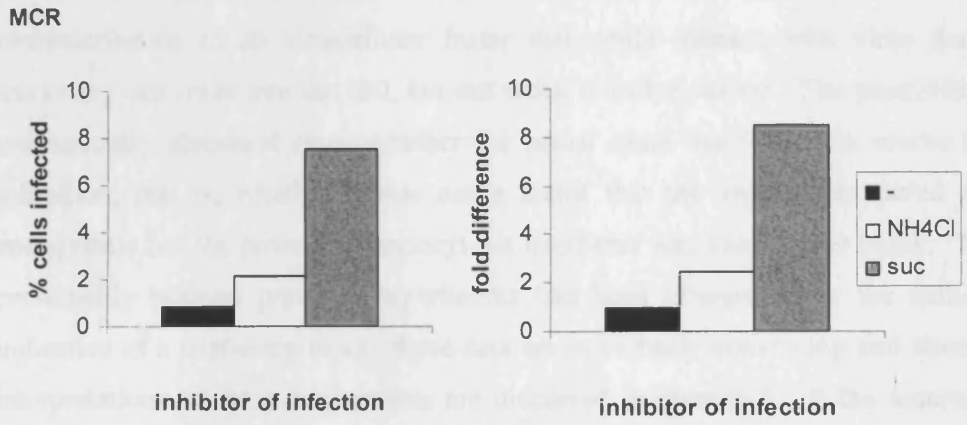


Fig. 6.4.2 Inhibition of entry by NH_4Cl or sucrose raises titres of MCR pseudotyped virions. HeLa CD4 cells were plated and infected at MOI 2 in DMEM, DMEM + 40mM NH_4Cl, or 0.45M sucrose. Fold-increases should be interpreted with caution, and qualitatively as the graph on the left shows; FACS titres for MCR with no endocytic inhibitor were below 1%, which is the reliable detection limit for flow cytometric analysis. Despite this, inhibition of endocytosis clearly raises titres considerably as the raw titres for NH_4Cl and sucrose were 2.1% and 7.5% respectively.

The data in figures 6.4.1 and 6.4.2 show that while 40mM NH_4Cl is sufficient to completely inhibit viral entry via VSV-G, it causes a very modest 2-fold increase in titres for MCR–pseudotyped virus. Hypertonic sucrose caused a 2.4-fold decrease in

titres of VSV-G, and increased titres of MCR-pseudotyped virus by over 8-fold in HeLa CD4 cells. Due to the toxicity of sucrose, cells could not be pre-incubated before addition of virus, and were only incubated with virions for 40 minutes. However, this makes the increase in MCR-pseudotyped viral titres all the more noteworthy, as the virions had less time to bind to the cells than in the positive control, which was incubated for 2 hours. Hypertonic sucrose does not affect the pH of cells, but inhibits receptor-mediated endocytosis (Heuser and Anderson 1989). It is difficult to reconcile this result with the suggestion from experiments by Reuter et al. that endocytosis plays no part in decreasing titres of MCR pseudotyped virions (Reuter *et al.* 2005). As both they and another group report that prevention of endosomal acidification increased titres of MCR pseudotyped virus by 10x (Reuter et al. 2005) and 5x (Marchant et al. 2005), it is tempting to speculate that the significant difference between the two experiments represents the difference between viral binding and binding of an antibody onto the receptor.

In both the previous studies of MCR described, the emphasis was on the characterisation of an intracellular factor that could interact with virus that was traversing one route into the cell, but not when it took a detour. The possibility was not seriously discussed as to whether the actual route itself was the source of the inhibition, that is, whether it was not a factor that the virus encountered during endocytosis but the process of endocytosis itself that was causing the block. This is presumably because previous experiments had been interpreted by the authors as indicative of a post-entry block; these data are not wholly convincing and alternative interpretations of these experiments are discussed in chapter 5. If the assumptions that the block to infectivity seen with MCR pseudotyped virions is caused by the presence of an intracellular factor (chapter 5) and is post-entry (chapter 5 and figures in 6.3) are abandoned, then a new explanation should be proposed. The simplest explanation with roots in cell biology should be considered first, that is, a trafficking defect. MCR is not able to initiate fusion at the cell surface at levels corresponding to a productive infection. If MCR is able to bind CD4 and can indeed bind it tightly (Reuter *et al.* 2005), then what is happening to the virus? Could the CD4 be taken up from the cell surface and trafficked down an endocytic route – not, as previously suggested, to encounter an intracellular anti-viral factor but instead to encounter the degradative activity of the lysosome? Thus raising the intracellular pH of the cell by

NH_4Cl could increase infectivity by two ways; by inhibiting trafficking pathways into the cell, or by blocking the degradative action of the lysosomes. In the first case, the virus would spend more time at the cell surface or en route along the pathways, with an extended chance to initiate fusion. In the second case, the virus would again have an extended time in which to initiate fusion, but from within the endocytic pathway. The lack of effect of bafilomycin A1 on titres of MCR pseudotyped virus is somewhat baffling, but would suggest that the former is more likely.

6.5 Blocking action of Rab5 causes a modest increase in productive infection of MCR Env HIV in HeLa CD4 cells, but has no effect in NP2* cells

For virions that are taken up by endocytosis, many can be tracked through the endocytic pathway by mutational analysis of the different Rab proteins. These data presented here (and (Marchant et al. 2005) show that MCR Env mediates entry via fusion at the cell surface, and strongly imply that the inability of HeLa CD4 cells to mediate entry via MCR Env is partly due to a block at this level. Additional evidence for a surface fusion event is found in studies of the effects of dominant negative Rab5 in NP2* cells, shown in the data in figure 6.5.1

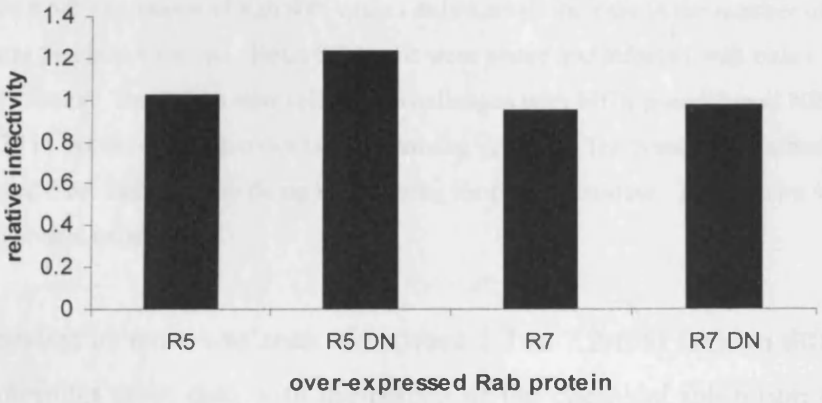


Fig. 6.5.1 Inhibition of Rabs 5 and 7 does not inhibit the entry of MCR pseudotyped NB-MLV into NP2* cells. NP2* cells were plated and transduced with Rab vectors detailed in the graph. Three days later cells were challenged with MCR pseudotyped NB-MLV. Infection was assayed by FACS 3 days later. Shown is the proportion of cells expressing the rab proteins that were infected by the challenge virus compared to negative cells. Results shown are from one experiment.

Dominant negative Rab5 inhibits transport to the early endosome. If endocytosis was a productive route of entry for MCR pseudotyped HIV in NP2* cells, then over-expression of Rab5 DN should reduce titres, as seen for VSV-G pseudotyped virions (chapter 3). Down-regulation of Rab5 appears, if anything, to slightly increase titres by 20%, however, supporting the case for productive entry via MCR Env as via fusion at the cell surface.

The experiment above could not be carried out in precisely the same way in HeLa CD4 cells, as titres are too low by FACS to give a sufficient number of positives cells for a background infection level to calculate the increase in titres. An older viral titre assay was therefore reverted to, and titres of MCR pseudotyped viruses delivering LacZ vector were assessed by counting fixed and stained blue cells.

	# blue cells			
	No virus	+virus	+virus, +Rab5DN	fold-increase
Expt 1	0	30	217	7.2
Expt 2	0	49	132	2.7

Table 6.5.2 Expression of Rab5DN causes only a small increase in the number of MCR pseudotyped viruses that infect the cell. HeLa CD4 cells were plated and infected with either Rab5DN or a mock (null) vector. Three days later cells were challenged with MCR pseudotyped NB-MLV carrying pHIT111 vector, which encodes lacZ-containing genome. The presence of infected cells can be assayed three days later by fixing and staining for β-galactosidase. Data shown were obtained in two independent experiments.

A modest increase was seen of between 2.7 to 7.2-fold for two different experiments. Combining these data with the results of the chemical inhibition of endocytosis and the microscopic fusion assay would suggest that the block to entry via MCR might be two-fold. First of all, virus is not readily binding to CD4. This is suggested by the much lower viral count from microscopy data. This could be either due to a lack of CD4 at the cell surface, or to the fact that as MCR is derived from a primary HIV-2 isolate, the envelope is ill adapted for rapid binding under conditions of *in vitro* cell culture. Secondly, once a few viral particles have bound, they are not successfully

initiating fusion and entry into the cytoplasm from the cell surface. Either MCR is binding too tightly (Reuter et al. 2005) or it is not initiating fusion in time to prevent CD4 endocytosis and destruction in the lysosomes. Preventing endocytosis by addition of inhibitors of endocytosis (chemical or biological) would therefore have only a small effect, as it would only affect that small percentage of virions that had successfully bound. It was therefore decided to investigate what factor(s) could be affecting CD4 expression at the cell surface.

6.6 Expression of $p56^{lck}$ renders HeLa CD4 cells permissive to MCR pseudotyped virions

CD4 is a marker of differentiation found primarily on T cells, and interacts with class II major histocompatibility complex antigens during antigen presentation. Expression is linked to the stage of positive selection that thymocytes have reached; they will pass through double negative, double positive, and finally singly positive stages for CD4 and CD8. As non-thymocytes, CD4 would not be expected to be expressed in the HeLa CD4, NP2* or U87* cells, however, so the regulatory mechanisms that would normally control CD4 expression and internalisation may also be awry or absent.

Control of CD4 levels at the cell surface is mediated by the tyrosine kinase $p56^{lck}$. $p56^{lck}$ is involved in T-cell development at several stages, including signalling to halt β-chain gene rearrangement, as well as playing a key role in signalling pathways after activation of CD4, in conjunction with components of the T-cell receptor. It has also been shown to be a key regulator of the endocytosis of CD4 (Pelchen-Matthews et al. 1991; Pelchen-Matthews et al. 1992; Pelchen-Matthews et al. 1995). In cells that express $p56^{lck}$, it is constitutively found associated with the cytoplasmic domain of CD4. $p56^{lck}$ itself has regulatory tyrosine residues, is activated by phosphorylation of the kinase domain and inactivated by phosphorylation of the carboxy terminus, which is reversed by the phosphatase CD45 during signalling. Dissociation of $p56^{lck}$ and CD4, and phosphorylation of two serine residues in the cytoplasmic domain of CD4 lead to its endocytosis (Pitcher et al. 1999). CD4 that has no cytoplasmic domain and cannot interact with $p56^{lck}$ is likewise constitutively endocytosed (Pelchen-Matthews et al. 1989; Pelchen-Matthews et al. 1995). As unsuccessful entry of MCR virus

could be due to either extensive endocytosis of receptor-virus complex before initiation of fusion can occur or low expression levels of CD4 at the cell surface, it would be worthwhile considering if $p56^{lck}$ could affect either of these contributions to the Lv2 phenotype.

Human $p56^{lck}$ was cloned into the vector pLgatewayIRESYFP, creating a vector expressing $p56^{lck}$, and YFP from an IRES. HeLa CD4 cells were transduced with the vector, then challenged with MCR pseudotyped virus. The effects of $p56^{lck}$ on titres are shown in figure 6.6.1.

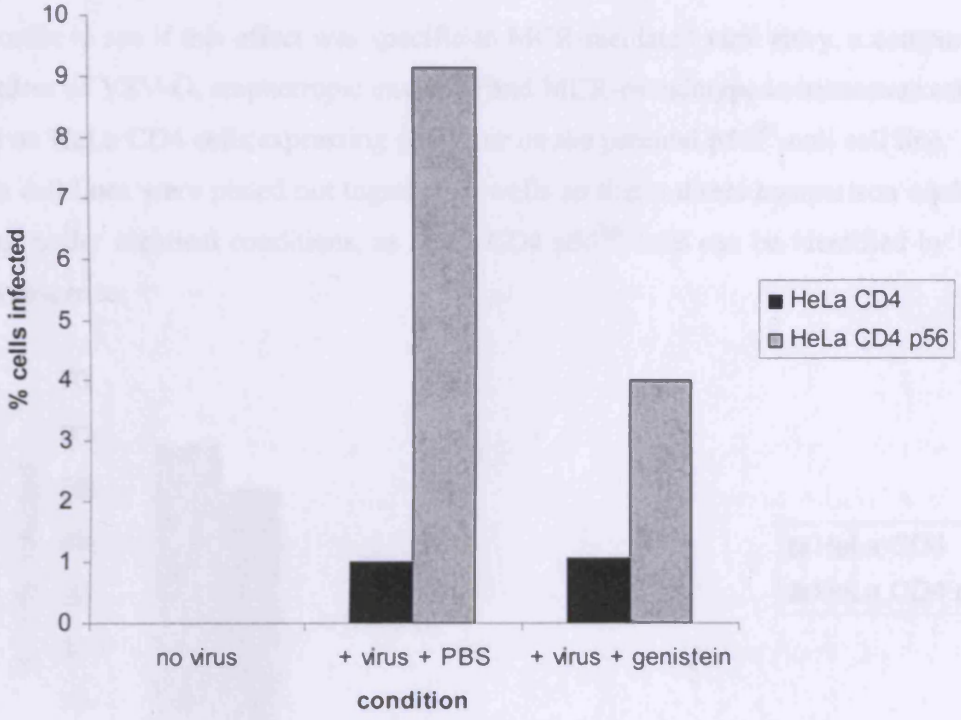


Fig. 6.6.1 Expression of $p56^{lck}$ renders HeLa CD4 cells over 10x more susceptible to MCR pseudotyped virus. $5x10^4$ HeLa CD4 or HeLa CD4 $p56^{lck}$ cells were plated, pre-incubated with PBS or genistein, and infected with MCR pseudotyped HIV-1 at MOI 1. Genistein is an inhibitor of tyrosine kinase, and would be expected to negate any effects of $p56^{lck}$ on titres. The medium/genistein/PBS was replaced after 2 hours, and the extent of infection was assayed 3 days later by FACS. Shown are the combined results of two experiments.

When $p56^{lck}$ is expressed in HeLa CD4 cells, titres increase dramatically from ~1% (i.e. at the limit of detection) to 9-fold higher. However, if the tyrosine kinase inhibitor genistein is added, titres are reduced. It was noticeable that the addition of

genistein had variable effects, possibly due to the extreme difficulty in dissolving the powder in aqueous solutions. The first experiment performed used a saturated solution, and reduced titres to back under 1%. In the second experiment performed the genistein had much less of a noticeable effect, but crystals of genistein were not seen amongst the cells indicating that the solution may not have been saturated. Either way, at over 10-fold, the increase in titres effected by expression of $p56^{lck}$ vector is considerable. Expressing $p56^{lck}$ in NP2* cells raises titres only two-fold, indicating that the cause of the block to infection that $p56^{lck}$ can overcome in HeLa CD4 cells does not affect NP2* cells.

In order to see if this effect was specific to MCR-mediated viral entry, a comparison of titres of VSV-G, amphotropic envelope and MCR-pseudotyped virions was carried out on HeLa CD4 cells expressing $p56^{lck}$, or on the parental $p56^{lck}$-null cell line. The two cell lines were plated out together in wells so that a direct comparison could be done under identical conditions, as HeLa CD4 $p56^{lck}$ cells can be identified by YFP fluorescence.

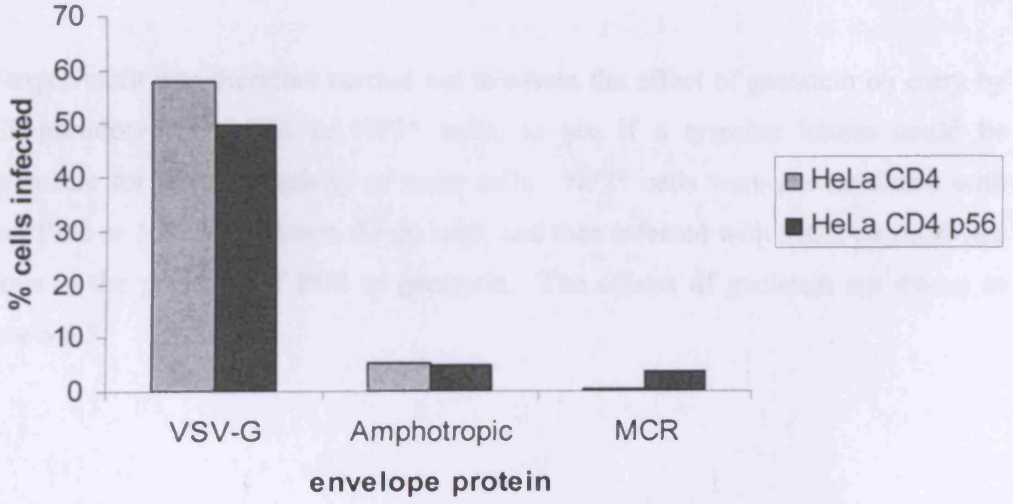


Fig. 6.6.2 A comparison of titres of HIV-1 virions pseudotyped with three different envelope proteins in HeLa CD4 cells, and HeLa CD4 cells expressing $p56^{lck}$. Expression of $p56^{lck}$ in HeLa CD4 cells does not alter titres of viruses pseudotyped with VSV-G or amphotropic envelope. In HeLa CD4 cells expressing $p56^{lck}$, titres are increased up to 10-fold (and see Fig 6.6.1)

While the expression of $p56^{lck}$ in HeLa CD4 cells did not change titres significantly for VSV-G or amphotropic envelope pseudotyped virions, titres were increased for MCR.

The addition of $p56^{lck}$ to HeLa CD4 cells results in an increased ratio of cell surface to intracellular CD4, and a decrease in the rate of endocytosis (Pelchen-Matthews et al. 1995). Neither of these should affect entry by VSV-G or amphotropic virus, and this was indeed the case, showing that the effect of $p56^{lck}$ is specific to CD4, and therefore MCR Env. Both of these aspects may help to explain part of the block to infectivity against MCR pseudotyped virions in HeLa CD4 cells.

However, the block to infection seen in HeLa CD4 cells is only part of the Lv2 story, and it must be further explained why this is apparently not the case for NP2* and U87* cells. These are astrocytoma cells, and as non-lymphoid in origin, would not be expected to endogenously express $p56^{lck}$. However, it has been reported that $p56^{lck}$ is not necessarily lymphoid specific, and has been detected in both rat and mouse neurons, albeit at low concentrations (Omri et al. 1996; Van Tan et al. 1996).

An experiment was therefore carried out to assess the effect of genistein on entry by MCR-pseudotyped virions to NP2* cells, to see if a tyrosine kinase could be responsible for the permissivity of these cells. NP2* cells were pre-incubated with either PBS or 100μM genistein for an hour, and then infected with MCR-pseudotyped virions in the presence of PBS or genistein. The effects of genistein are shown in figure 6.6.3.

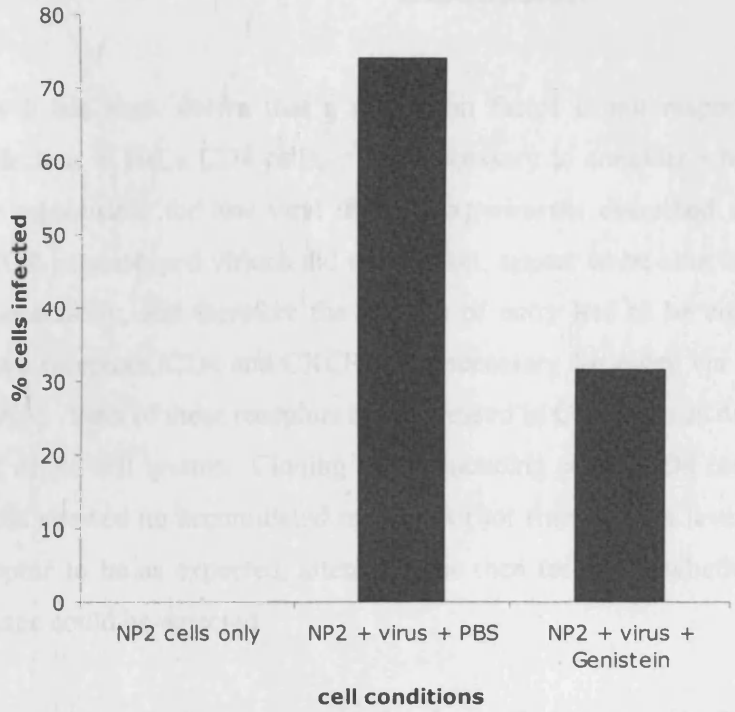


Fig. 6.6.3 A tyrosine kinase inhibitor, genistein, reduces titres of MCR pseudotyped virions by half in NP2* cells. Cells were infected as described in the legend to figure 6.6.1. Shown are the results of one experiment. In a separate experiment, no decrease in titres on addition of DMSO or ethanol (solvents of genistein) was seen (not shown).

When NP2* cells were incubated with genistein before infection with MCR-pseudotyped virus, titres were reduced by 50%. This would imply that the difference between NP2* and HeLa CD4 cells in their permissivity to MCR Env pseudotyped virus may be partly, but not solely related to increased surface expression of CD4 alone. In fact, even when expressing high levels of $p56^{lck}$, HeLa CD4 cells were still significantly less permissive than NP2* cells, for example, the data in figure 6.6.1 shows that titres on HeLa CD4 $p56^{lck}$-expressing cells reach 10%; titres on NP2* cells with the volume of virus used in this experiment typically reach over 70% cells infected. There will be other reasons why HeLa CD4 cells are so refractory to infection by MCR Env pseudotypes, and these effects combine to reduce titres so drastically compared to NP2* cells.

Discussion

As it has been shown that a restriction factor is not responsible for the block to infection in HeLa CD4 cells, it was necessary to consider what other factor(s) could be responsible for low viral titres. Experiments described in chapter 5 show that MCR-pseudotyped virions did not, in fact, appear to be able to enter HeLa CD4 cells successfully, and therefore the process of entry had to be considered in these cells. Two receptors, CD4 and CXCR4, are necessary for entry via MCR (McKnight et al. 1998). Both of these receptors are expressed in CD4 cells as detected by Western blot of whole cell lysates. Cloning and sequencing of the CD4 receptor from HeLa CD4 cells showed no accumulated mutations (not shown). As levels of CD4 and CXCR4 appear to be as expected, attention was then turned to whether a defect in receptor usage could be detected.

In order to do this, a recently developed fusion protein, S15-mCherry was used. When expressed in producer cells that synthesise virus, S15-mCherry labels the viral membranes. As can be seen from the pictures and the data in sections 6.2 and 6.3, this label provides a method of assessing membrane fusion, the first stage of a productive infection. The data clearly show that MCR pseudotyped virions are not fusing with HeLa CD4 cells, and in fact also suggest that a reduced absolute number of virions (about a quarter and a half of the number of virions compared to NP2* and U87* cells respectively) is binding to HeLa CD4 cells (see appendix 3).

If successful fusion is not taking place, this could be due to several factors. The virions could themselves be defective, but this is unlikely given the high titres obtained with the same batch of virus on NP2* and U87* cells. There could be a problem with CD4 expression or processing in HeLa CD4 cells, but this is also unlikely given that it was detected in a Western blot of whole cell lysates, and HeLa CD4 cells are routinely used in viral titre assays for CD4-tropic virus. Alternatively, it could be that CD4 is not found at high concentration at the cell surface, due to rapid endocytosis, and that when it is trafficked out to the cell surface, the maladapted MCR is not able to bind with enough rapidity before CD4 disappears again. Another explanation is that MCR is binding, and binding too tightly (as suggested by Reuter et

al. (Reuter et al. 2005)), and is not initiating fusion at the cell surface before the virus-CD4 complex is endocytosed. This is not a productive entry pathway for HIV. The small increase in titres in HeLa CD4 cells in which Rab5 is down regulated, or acidification of endosomes prevented, support the suggestion that endocytosis plays a role, albeit along with other factors.

It should be borne in mind that MCR was derived from a primary isolate, and therefore may be adapted for physiological conditions rather than the tissue culture conditions described in these experiments. Certainly the cells that the MCR envelope would be interacting with in an *in vivo* infection would be cells of lymphocytic origin, which carry a plethora of regulatory mechanisms to ensure CD4 remains at the cell surface. These mechanisms are thought to be largely lacking in cells of non-lymphocytic origin. Experiments described here in which titres of MCR pseudotyped virions are increased over 10-fold by expression of $p56^{lck}$ support the suggestion that lack of CD4-regulatory mechanisms in HeLa CD4 cells are an important part of the reason for low titres. Within the physiological context, tight binding of MCR Env to CD4 on lymphocytes would probably be advantageous in the harsh extracellular environment, and as $p56^{lck}$ would ensure that CD4 is present at the cell surface, the likelihood of this tight binding leading to undesired endocytosis is lowered.

With this hypothesis in mind, the question remains why NP2* and U87* cells, also of non-lymphoid origin, are apparently able to maintain CD4 at the cell surface. It has been suggested that $p56^{lck}$ is expressed in rat and mouse brains (Omri et al. 1996; Van Tan et al. 1996). Preliminary Western blots of NP2* and U87* cells gave a tantalising hint that there could be miniscule level of $p56^{lck}$ protein in these cells (not shown), but this does not preclude another CD4-regulatory mechanism being active as well. Different cell lines from the same species can have utterly different intracellular pathways, optimised for their particular role in the organism, and experiments in cell biology and trafficking in one cell type cannot be presumed to reveal generalities for all cell types from that species. The fact that MCR pseudotyped virions are able to infect NP2* and U87* cells successfully should not be taken as evidence that they are able to infect all human cells, but simply that they are competent for infection *per se*, and in some cell lines the infection is successful.

In March 2007, towards the end of this period of work, a new restriction to infection was described that also has a route of entry component, and dubbed Lv3 (Pineda et al. 2007). A rhesus macaque epithelial cell line expressing human CD4 (sMAGI) exhibited two levels of post-entry restriction to HIV-1, the first level being Trim5α. The second level was operative against HIV-1 core pseudotyped with SIV envelope, or an envelope from an R5 clone, SF162. The cells were not infected even at the highest doses, indicating that the block to restriction was not caused by a saturable factor, and addition of SiRNA against Trim5α had no effect on titres. The block in sMAGI cells could be circumvented by pseudotyping with VSV-G, or when sMAGI cells expressed human CCR5, if Trim5α was also abrogated. A threshold of virus entry which was detectable by qPCR data (~25 copies) was reached at 1-2ng/ml Gag; at all doses levels were ~100x lower in sMAGI cells compared to sMAGI-CCR5. Levels of the early products of reverse transcription can be usefully used as markers of viral entry when there is a significant increase over baseline levels. However, as some reverse transcription can occur in the retroviral particle pre-entry low levels of reverse transcription products cannot be reliably assumed to be solely due to post-entry events.

For example, on non-permissive sMAGI cells, 2ng/ml Gag equates to an MOI of ~0.32, and 50 copies of early RT products per 20,000 cells. The highest level that is reached is 1000 copies per 20,000 cells at 40ng/ml i.e. MOI of ~6.4. Considering that there is such a high MOI of virus laid on the cells, the number of copies per cell seems unreasonably low, if the desired point to be derived from the data was that entry was uninhibited (compare to the 100x higher levels in the unrestricted sMAGI-CCR5 cells). Cells were washed before analysis which would suggest that the low-level reverse transcription that occurs in free virions could not be responsible (Trono 1992; Zhang et al. 1993). However, in this particular experiment virions would not necessarily be readily removed by washing even if they were not able to enter the cell. If the block to infection were indeed at entry due to lack of an optimised co-receptor, virions would still bind to CD4 and thus be resistant to removal by washing. It is conceivable that the reverse transcription products detected from the sMAGI cells are, therefore, due to reverse transcription occurring in these bound extracellular virions.

A cell-cell fusion assay to test the efficiency of fusion of the endogenous rhesus co-receptor revealed a part of the block, and concluded that SF162 envelope was reduced in efficiency for fusion with sMAGI cells compared to cells expressing CCR5, although the relevance of cell-cell fusion for assessment of viral envelope viability is not necessarily confirmed. Another viral envelope from SIV, however, was equally efficient for fusion on both cell lines, but was similarly restricted during the infectious cycle on sMAGI cells, again by a block that was unsaturable by nature. It was not reported how levels of early RT products compared for this envelope.

In order to be completely certain that a block was post-entry it would need to be ascertained that fusion and entry of the virus were uninhibited, and at comparable levels to unrestricted virions. For example, for $Fv1^n$ this was done by comparing levels of both early and late reverse transcription products for N-(unrestricted) and B-(restricted) tropic MLV. Regarding rhTrim5α, levels of early products of restricted and unrestricted virus were not comparable as the block is pre-reverse transcription, but the saturable nature of the block overwhelmingly indicated that a post-entry, cytosolic restriction was involved, rather than a block at the level of fusion and entry. For Lv3 there is more uncertainty that fusion is sufficient with the SF162 envelope and the putative rhesus co-receptor (expressed endogenously on sMAGI cells), and comparisons between titres on sMAGI and sMAGI-CCR5 cells are only questionably relevant. Certainly the sMAGI cells engineered to express the human CCR5 may artificially increase the levels of virions entering the cells by virtue of the over expression of co-receptor, but this would simply reflect the difference between cells able to take up artificially large levels of virions and cells with levels of viral entry that have not been thus amplified, rather than revealing a bona fide restriction.

In conclusion, HeLa CD4 cells are poorly infectable by virions pseudotyped by MCR Env but this is primarily due to inhibition of the viral lifecycle at the level of entry, to a combination of poor availability of CD4 at the cell surface and sub-optimal interaction of MCR Env and CD4/CXCR4, rather than a post-entry block caused by a specific anti-viral factor. While the type of block to infection suggested by Lv2 as a route of entry-dependent restriction is not by any means impossible, it is intuitively unlikely, and experiments in order to uncover a post-entry restriction using MCR are difficult, given the overwhelming effect of the entry block. In order to reverse

transcribe over a baseline level, virions must access the pool of dNTPs in the cytoplasm. If they have reached the cytoplasm and reverse transcribed, the idea that they would then somehow re-enter a subcellular compartment (i.e. one in which an anti-retroviral such as Lv2 might be active) in order to access the nucleus is not probable based on current knowledge. It is not, however, impossible that a factor could bind to the virion as it passed through various endocytic compartments on its way into the cell cytoplasm, and that this factor would only manifest an effect later on in the viral lifecycle. This would then be in effect a route of entry-dependent restriction, as it would depend on the route that the virus took to get into the cytoplasm even though the block was later on. For HeLa CD4 cells and MCR Env, however, this is manifestly not the case, as the virus is not even entering the cell.

Chapter 7

Thesis Discussion

Rationale

During these studies, the overarching aim has been to shed light on the earliest events of retroviral replication. It is unfortunate that the commonest methods of detecting successful entry rely on all stages of the early life cycle being completed, and the virion being able to integrate successfully. Thus, from an initial FACS assay, an entry block appears very similar to a block at integration as in both cases no fluorescent signal is produced. However, in the former the virus has not even penetrated the cell, whereas in the latter, the virus has entered, reverse transcribed and accessed the nucleus. Other techniques must be employed to fill in the missing details. In this thesis a variety of methods were used both to try and control variations in the early stages of the lifecycle, and also to detect success at each of these stages. For example, different combinations of envelope proteins and receptors were used to try and direct the virus into the cell by different routes, and the success of attempted entry of the virus monitored by tracking loss of a fluorescent signal from a protein in the viral membrane. These techniques aid localisation of blocks to replication more precisely in both time and the space of the cell and the infection process.

The characteristics of the entry pathways mediated by the Tva800 and Tva950 after binding of ASLV Env were investigated. The differences between the two pathways were probed using chemical and microscopic methods, and an attempt made to assess how these differences might be manifest.

During the course of these studies, a novel titration curve obtained using Tva800 as a receptor for viral entry inspired a series of experiments designed to clarify quantitative aspects of viral binding and fusion.

A previously described block to replication, Lv2, was re-examined and the principles of this phenomenon (namely that variation in route of entry into the cell can have a profound effect on subsequent steps of replication) were assessed.

Results

It was found that Fv1 and Trim5α restriction did not have a route of entry dependence that was revealed by these experiments. This is intuitively what would be expected, as restriction factors do not act on whole virus, but after the membrane has been lost, on the incoming core. Irrespective of the current debate over whether Trim5α effects restriction via a more rapid disassembly or via degradation of the core by another method, it is difficult to conceptualise how any binding sites would be revealed until the viral membrane has been lost and the core delivered into the cytoplasm. Any variations taken in the pathway up to that point of fusion would, therefore, be irrelevant, as long as the virus did ultimately reach it. Hence, although Tva800 and Tva950 appear to traffic the virus into the cell by slightly different routes, they both deliver the virus to a functional entry point (the late endosome?) at which fusion can occur. After the virus is delivered to the cytoplasm, subsequent actions on the core would affect those that entered via Tva800 and Tva950 equally, and this is what was found with the experiments described here with Fv1 and Trim5α. For these two restriction factors, it is probable that there is no route of entry component.

The routes that virions take after binding to Tva800 and Tva950 remain to be fully described. Differences in the susceptibility of the virus to degradation under an NH_4Cl-induced block to infection imply that either the micro-location of the receptor, the structure of the receptor or the different routes that the receptor directs the virus down after binding have a profound effect on subsequent events. GPI-linked receptors located in lipid rafts are thought to direct ligands down caveolae-mediated endocytic routes which have slower kinetic parameters than clathrin-mediated endocytosis, and direct to caveosomes, caveolin-1 containing endosomes. The lipid raft areas of the membrane are enriched for cholesterol, glycosphingolipids and sphingomyelin, which collude to stiffen the membrane. During endocytosis the membrane will invaginate and pinch off, travelling into the cell, and the caveosome dynamics may be affected by the composition of the membrane it has been formed from, namely the lipid raft area. Lipids themselves play key roles in the targeting of intracellular organelles and in fusion dynamics, for example, phosphoinositide 3-phosphate is an important targeting signal for endosomal traffic (Hurley and Meyer

2001). In itself, it is not enough to target an early endosome; Rab5 is needed, but is part of the overall recognition of the docking site. The bulkiness of the lipids forming the caveosome could have a profound effect on targeting and fusion dynamics of the caveosome, the release of ligands bound at the cell surface, on viral penetration of the caveosome membrane to enter the cytoplasm, and could be a key feature in the different viral entry kinetics and susceptibility to NH_4Cl for Tva800 and Tva950. All this is speculative, but as the binding domains of the receptors are identical, and clearly the envelope protein of the virus is likewise, so the functional difference in the two receptors must relate somehow to their attachment and localisation.

The local environment of Tva800 or Tva950 in the cell membrane may also explain a further difference between the two receptors, namely the requirement that a virus binds >1 Tva800 receptor in order to be able to enter a cell successfully, but that binding of only one is necessary for entry via Tva950. Entry of the virus requires a considerable input of activation energy to perturb the cell membrane sufficiently; the decreased fluidity of the cell membrane in lipid rafts may require the extra energy input provided from extra receptor binding and fusion activation in order to overcome the extra activation energy required for the stiffer membranes.

Virions carry multiple copies of envelope proteins, although there is considerable debate both about precisely how many extra copies they carry, and whether all of these are functional or not. Chertova et al. reported that HIV-1 virions contained Gag:Env ratios that corresponded to between 7-14 trimers per virion, although the simple presence of a trimer is no guarantee that it is functional (Chertova *et al.* 2002). Using antibody neutralising studies, Yang et al. concluded that only one functional trimer is necessary to mediate viral infectivity for HIV-1, amphotropic MLV and ASLV-A (with Tva950) (Yang *et al.* 2005a; Yang *et al.* 2005b). ASLV-A entry with Tva800 was not studied (Yang *et al.* 2005b). Relative infectivities of virions possessing different ratios of dominant negative mutant Env to wild-type Env were plotted, and compared to the curve expected from a model where one functional trimer is needed for infectivity. From another study by the same authors it was furthermore concluded that only two wild-type units within a trimer were required for that trimer to be functional (Yang *et al.* 2006).

However, these findings have been disputed with alternative accounts of the data, which primarily address key assumptions that interpretation of the experimental data relied on. For example, a key assumption is that the number of infectious virions in a stock is as low as the number that actually infect cells under normal conditions. But viral titres can be raised when interactions with cells are increased, as infectious virions are lost to unproductive interactions with the cell or are endocytosed. Thus the small percentage that infects is not the same as the proportion that is infectious. The assumption that each virion only has the minimal number of trimers required for infection is also unsubstantiated, as functional Env may not be the dominant limitation of infection. Klasse carried out a mathematical investigation of two types of models, in which the number of Env trimers required for infection is either over a minimum threshold or whether interaction of each successive trimer incrementally raises the chance of a successful infection (Klasse 2007). The most likely scenario that best-fit models suggested is a combination of these two possibilities, which could vary for different viruses, and mutations of Env. Models explored the redundancy of Env protomers to generate a functional trimer unit, and the number of functional trimers required for a functional virion. It could be the case, for example, that rather than an all-or-nothing scenario in which 2 non-functioning protomers render a trimer inactive but 1 non-functioning protomer does not, it would be much more likely that each protomer adds to the probability of a trimer being functional, but a virion with (for example) 7 semi-functional trimers is much less likely to be infectious than one with 7 fully-functional trimers, hence the threshold levels of activation come into play. A model to demonstrate this, however, may not be readily distinguishable, especially as both fully and partially functional trimers may contribute to cell binding, if not to infectivity.

In short, the best interpretation is that differences between virions and Env mutations contribute to a blurring of potential models, but that a combination of incremental effects of each protomer to trimer function exists, and an increasing number of functional trimers eventually take a virion over a real threshold of activation (Klasse 2007). This threshold cannot be equivalent to 1 trimer, as other data rule out this option. For example, the phenomenon of antibody-dependent neutralisation described for dengue virus and HIV-1 (Schutten *et al.* 1995; Goncalvez *et al.* 2007) rules out the possibility that a virion could have only one trimer (functional or not). This

phenomenon describes how increasing concentrations of antibody against envelope increase the proportion of virus entering a cell up to a point, after which further increases in antibody block entry. It is thought that up to the turning point, antibody against envelope neutralises negative charges on the virion, which would exert some level of repulsion away from the cell surface, so increasing viral titres. After the turning point, however, addition of further antibody starts to neutralise the viral envelope protein to the extent that the virion is no longer able to initiate binding and entry. So this explanation requires that in addition to the envelope protein(s) that mediate entry, at the very least the virion would require additional trimers sufficiently functional as to be capable of binding antibody.

Studies of HIV are complicated by the requirement of receptor and coreceptor, and additionally by the interdependence of levels of CD4 and CCR5 or CXCR4. However, studies done argue against formation of an entry complex and virion pore mediated by a single trimer as HIV-1 could initiate infection with low levels of CCR5 co-receptor if levels of CD4 were sufficiently high (or vice-versa), and that between 4-6 CCR5 molecules were required to bind the virion (Kuhmann *et al.* 2000).

Within this framework, the data presented in chapter 4 are not surprising. The concept of multiple receptor binding events being required for successful virion entry is one that sits easily with the current models and data for other viral envelope-receptor pairs. The simplicity of the ASLV Env, Tva800 and Tva950 system lends itself as a model system to facilitate further studies in this area. The interaction does not require any additional factors or coreceptors, which simplifies things considerably. Additionally, the mechanism of entry is via endocytosis, which means that there are no issues with virions being considered non-infectious when they have simply embarked on a non-productive route of entry, as is found for virions with a requirement for fusion at the cell surface that get endocytosed.

All of the studies presented in this thesis have proceeded from the basic premise that the basic requirements of a virion and a cell, expressing the cognate receptor, are not necessarily a sufficient prelude to infection. However, it is also undoubtedly true that not all barriers to infection necessarily have anything to do with restriction factors. To give a well-known example, HIV-1 is able to initiate fusion into the cell from

receptors at the cell membrane. If, however, the virion starts down the endocytic pathway then the infection is unproductive as in most cell types it will be degraded in the acidic environment. This is a major reason for the extremely low particle: infectivity ratio for HIV-1. However, this does not in any way fall under the category of restriction as the virus is simply not managing to penetrate the cell successfully, and there is no specific factor that is acting on the virus and blocking replication. As the search for anti-retroviral agents continues, the need for new ways to counteract infection grows, but this should not warp perspectives so much that cellular and viral mismatches are not first considered thoroughly, before a novel mechanism is proclaimed.

That said, the possibility should not be discounted, however, that among the myriad of cellular compartments that a virus could pass through on its way into the cell, there could be those that contain anti-viral proteins or conditions that would irreversibly alter the virus. Although the virus may be able to proceed a little further in the infectious cycle, it is rendered unfit for completion. An example of something along these lines would be the Trim5α-mediated restriction of HIV-1 with and without the influence of MG132, a proteasome inhibitor (Anderson *et al.* 2006). When MG132 is absent, the viral infectious cycle and reverse transcription are both inhibited. When it is present, reverse transcription occurs so that products of it are found, but overall the infectious cycle is still blocked.

There is so much about the early stages of retroviral infection that remains to be understood, and cellular factors used by the virus that remain to be identified. A list of host proteins involved in HIV-1 infection was identified through a large scale SiRNA screen, and published towards the end of writing of this thesis (Brass *et al.* 2008). The authors identified over 250 host proteins involved at all stages from entry to exit, many of which were not previously known to be associated with HIV-1 infection. Further analysis of the proteins listed that were involved in stages of infection between entry and integration would be an excellent starting point in a search for restriction factor-like agents, or potential targets for anti-virals.

Conclusion

Finally, it is evident that Fv1 and Trim5α are merely forebears of what may be a whole range of intracellular anti-virals, and many more are sure to be discovered. While these offer potentially vast rewards in terms of information revealed about the viral life-cycle, and the workings of the cell, several criteria must be satisfied for a block to be considered both a restriction and significant in terms of potential therapeutics. Firstly, a significant decrease in viral titres must be evident. Previous work assigned the term restriction only to decreases of 100-fold or more in viral titres (Hartley *et al.* 1970). A decrease of less than 10x must be viewed with extreme scepticism to ensure that different cell or experimental conditions are not causing a variation in titres consistent with experimental error. Secondly, before putative restriction factors are invoked it must be proven beyond any reasonable doubt that the virus is indeed entering the cell at unrestricted levels, and that the block to infection is due to a process significantly downstream of entry. This could be done by detection of later products of reverse transcription, or alternatively by abrogation of the block. Thirdly, the block should ideally be relevant on a physiological level. For example, rhesus macaques were shown to be refractory to infection by HIV-1 long before the discovery of Trim5α, and the first discoveries about Fv1 were made using whole mice, rather than the cell lines. If at least the first two criteria are satisfied then a block to infection may be worth pursuing, and classifying as a restriction.

Further Work

During the course of the work described in this thesis many questions were raised, some of which remain unanswered. The nature of the differences between Tva800 and Tva950 has been partly clarified, yet the way in which these differences affect the routes into the cell taken from the receptors is still not clear. Ideally further tracking studies could be done to trace virions as they pass into the cell, potentially highlighting other important intracellular compartments. The use of a broad range of dominant negative inhibitors would also aid characterisation.

In the studies of how many Tva800 receptors are needed for viral entry, a more quantitative system was sought throughout. A tetracycline expression system,

whereby expression of a gene is controlled by the level of this antibiotic in the medium was made and tested, but was not functional in time for experiments to be included in this thesis. Further experiments in this area must generate more quantitative data, and a precise assessment of numbers of Tva800 at the cell surface in further studies is needed.

Further experiments to investigate the refractory nature of HeLa CD4 cells to infection by some CD4-tropic viruses, e.g. MCR, would have to compare HeLa CD4 cells to cells that would express CD4 in a physiological context. A thorough investigation of mechanisms that normally regulate CD4 cell-surface expression in lymphocytic cells is called for, with a view to understanding how these could be potentially be present in NP2* and U87* cells, even though non-lymphocytic in origin.

All of these would contribute significantly to the body of knowledge that must be built up in order to better understand aspects of retroviral infection that could be targeted in future therapies.

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Appendix 1

A ratio can be calculated that relates the absolute number of receptors on a cell and the absolute number of infectious virions hitting that cell as follows:

no. receptors[a] : inoculum[b]

[a]abs. no. receptors calculated by relative no. (1, 5, 10, 20)*preset ratio – 1.5

[b]inoculum = ul used*no infectious virions ul^{-1}

Next assumption: the availability of receptors necessary for an interaction on the target cell surface is given by a Poisson distribution, dependent on the above ratio

$$\sum_{k=0}^{\chi} \frac{e^{-\lambda}\lambda^{\wedge}}{k!}$$

Or, in Excel, POISSON[theorised no receptors, ratio, TRUE]

The proportion of **cells** with available receptors is
1-(sum of these terms) for each rec:virion ratio
ie 1-Σ(Poisson terms 0...r-1)

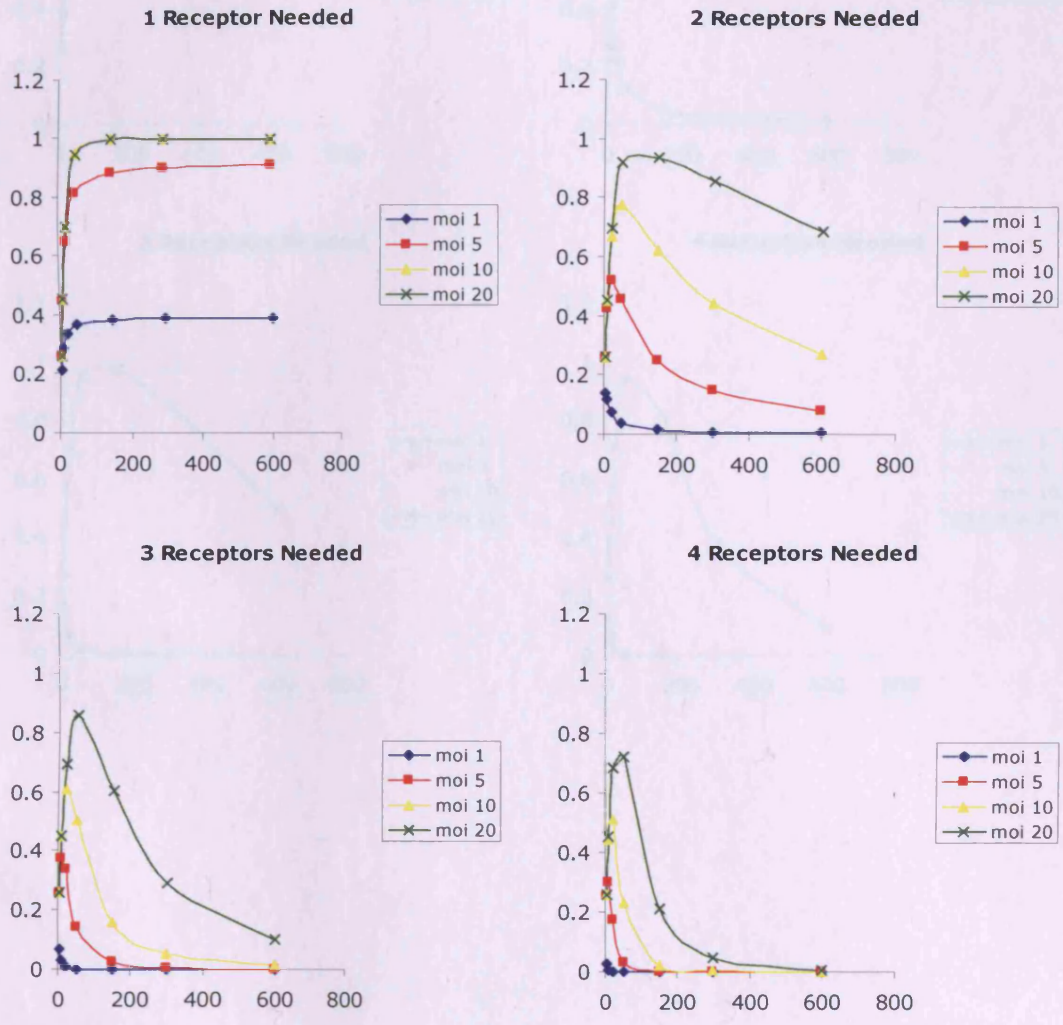
Therefore the proportion of **infected cells** is:

$1-e^{(\text{MOI GFP*prop. of cells with available receptors})}$

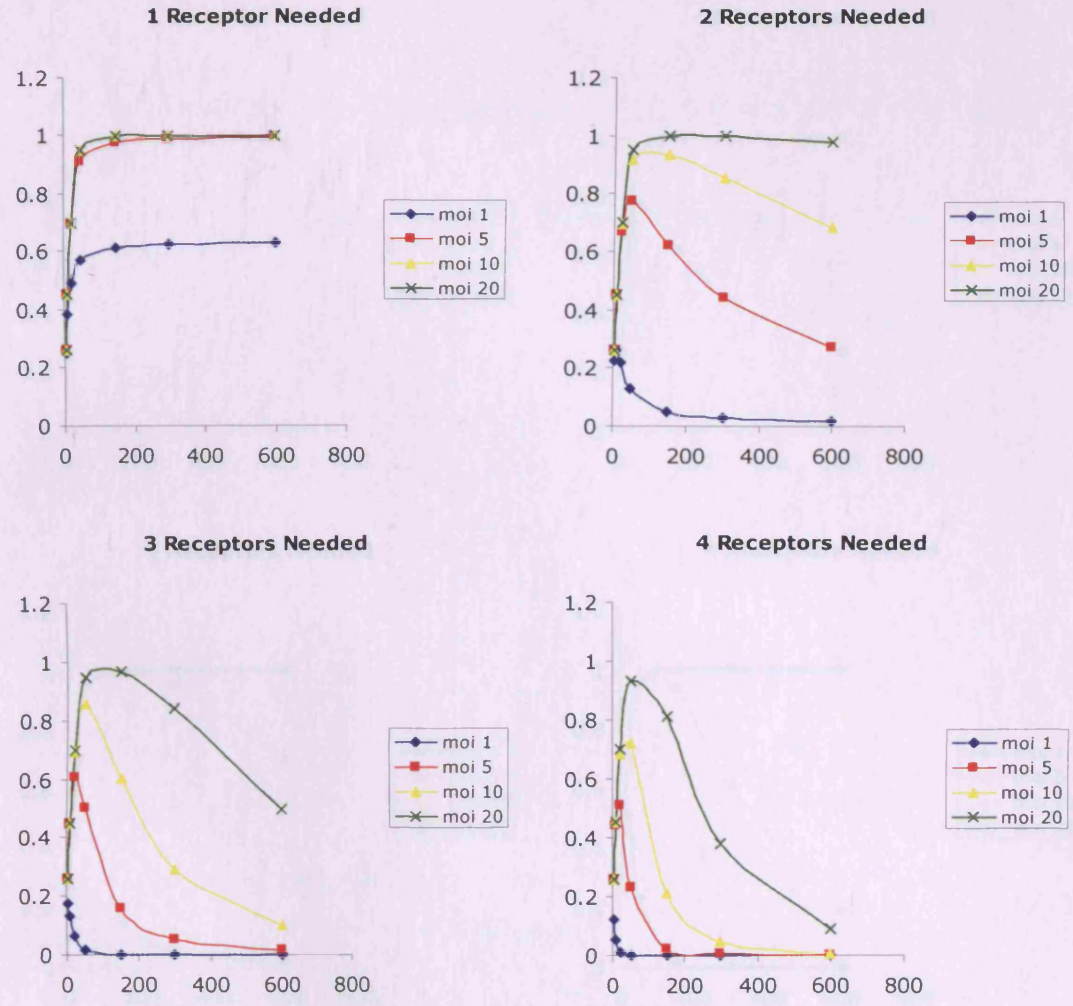
Appendix 2

Illustration of the shapes of graphs obtained if the ratio between the number of receptors and MOI YFP is varied between 0.5 and 5.

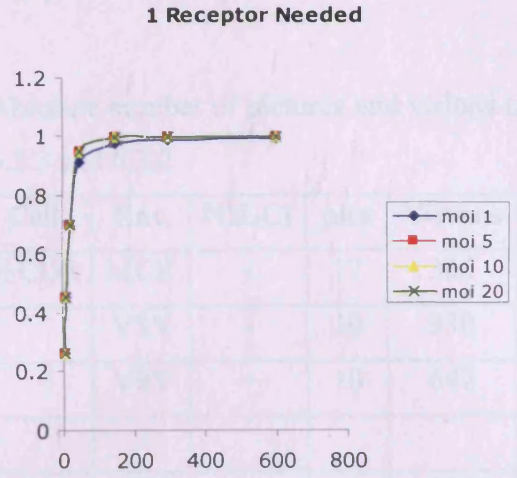
Ratio: 0.5

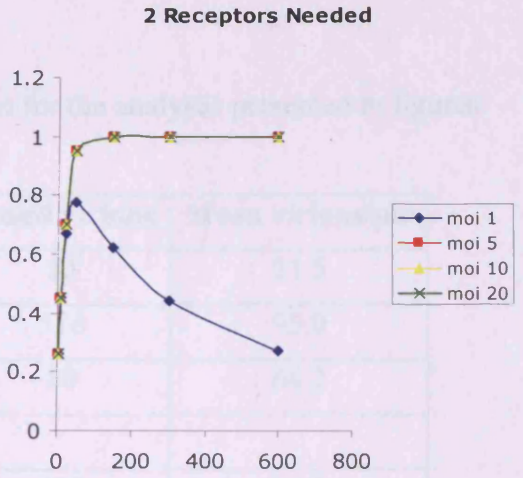


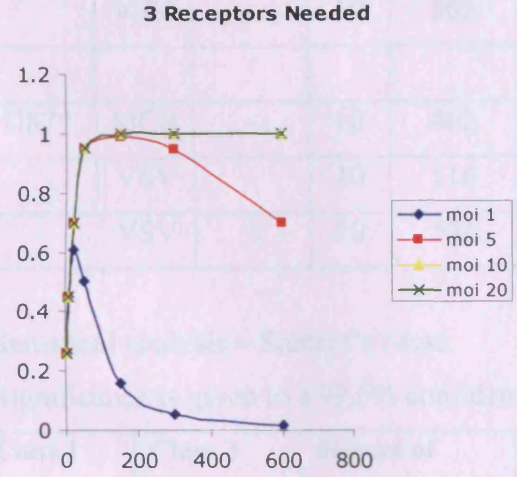
Ratio 1.0

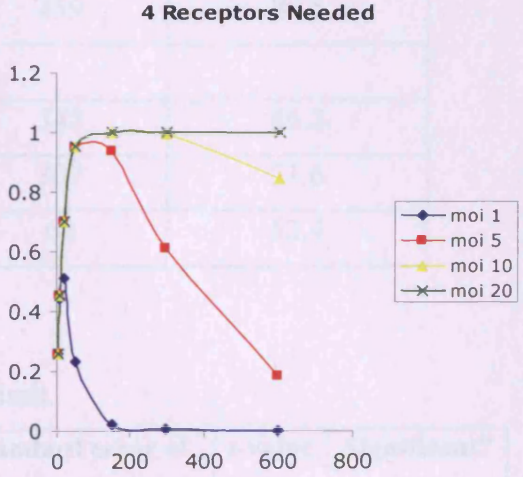

1 Receptor Needed
2 Receptors Needed
3 Receptors Needed
4 Receptors Needed
1.2
1
0.8
0.6
0.4
0.2
0
0
200
400
600
800
moi 1
moi 5
moi 10
moi 20

Ratio 5.0


1 Receptor Needed
1.2
1
0.8
0.6
0.4
0.2
0
0
200
400
600
800
moi 1
moi 5
moi 10
moi 20


2 Receptors Needed
1.2
1
0.8
0.6
0.4
0.2
0
0
200
400
600
800
moi 1
moi 5
moi 10
moi 20


3 Receptors Needed
1.2
1
0.8
0.6
0.4
0.2
0
0
200
400
600
800
moi 1
moi 5
moi 10
moi 20


4 Receptors Needed
1.2
1
0.8
0.6
0.4
0.2
0
0
200
400
600
800
moi 1
moi 5
moi 10
moi 20

Appendix 3

Absolute number of pictures and virions taken for the analyses presented in figures 6.2.3 and 6.3.2

Cell	Env	NH_4Cl	pics	Virions	Fused virions	Mean virions/pic
H CD4	MCR	-	17	366	55	21.5
	VSV	-	10	930	576	93.0
	VSV	+	10	642	80	64.2
NP2*	MCR	-	10	817	488	81.7
	VSV	-	10	805	459	80.5
U87*	MCR	-	10	462	345	46.2
	VSV	-	10	516	307	51.6
	VSV	+	10	524	62	52.4

Statistical analysis – Student's *t*-test.

Significance is given to a 99.5% confidence limit.

Class 1	Class 2	degrees of freedom	Standard error of difference between means	*t*-value	Significant?
HCD4 V	HCD4 V + NH_4Cl	18	0.0390	12.7	✓
U87* V	U87* V +NH_4Cl	18	0.0490	9.72	✓
HCD4 M	NP2* M	25	0.0629	-7.11	✓
HCD4 M	U87* M	25	0.0484	-12.3	✓
NP2* M	NP2* V	18	0.0658	0.412	×
U87* M	U87* V	18	0.0613	2.48	×
HCD4 M	HCD4 V	25	0.0456	-10.3	✓
HCD4 M	HCD4 V + NH_4Cl	25	0.0305	0.841	×